REMARKS

Claims 32-54 were pending in the application. Claims 33-39 were withdrawn from consideration as being directed to non-elected subject matter. Claims 51-54 have been cancelled. Claims 32, 42, 43, 46, and 47, have been amended. New claims 55 and 56 have been added. Thus, upon entry of the foregoing amendments, claims 32-50, 55, and 56 are pending in the application.

Support for the amendments to claims 32, 43, and 47, can be found throughout the specification, including at least at page 15, lines 21-25 and page 21, lines 4-7. Support for new claims 55 and 56 can be found throughout the specification, including at least at page 15, lines 25-27.

No new matter has been added. Applicants request that the amendments and cancellations to the claims be entered. The foregoing claim amendments and cancellations should in no way be construed as an acquiescence to any of the Examiner's rejections and were made solely to expedite prosecution of the present application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

Withdrawal of Rejection of Claims 32 and 40-50 Under 35 U.S.C. 112, Second Paragraph

Applicants gratefully acknowledge the withdrawal of the rejection of claims 32 and 40-50 under 35 U.S.C. 112, second paragraph.

Rejection of Claims 32 and 40-54 Under 35 U.S.C. 112, First Paragraph

Claims 32 and 40-54 are rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the enablement requirement. The Examiner states that the "claim(s) contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains...to make and/or use the invention."

Applicants respectfully traverse this rejection and request reconsideration.

As amended, the claimed invention describes a non-human transgenic animal comprising a transgene comprising a nucleic acid molecule encoding a fusion protein which activates transcription of a gene of interest operatively linked to a target DNA sequence to which the fusion protein binds, the fusion protein comprising a first polypeptide comprising a DNA binding domain operatively linked to a second polypeptide comprising a transcriptional activation domain, wherein the transcriptional activation domain comprises at least one copy of a mutated acidic region of herpes simplex virus virion protein 16 (HSV VP16), the mutated acidic region consisting of amino acid positions 436 to 447 of HSV VP16 (SEQ ID NO: 1) and having an amino acid substitution at position 442 as compared to wild type HSV VP16, the transgene being expressed in cells of the transgenic animal at a level sufficient to produce amounts of the fusion protein that can activate transcription of the gene of interest ***at detectable levels***.

The Examiner states that,

> ...the rejection is based on how to make and use the invention in commensurate with the scope of the claim. The phenotype of the claimed transgenic animal is essential to the enablement of the claimed invention because one skilled in the art would not know how to use a transgenic animal with claimed genotype, but shows no phenotypic feature. However, it is such phenotypic features in any given species of a transgenic animal which cannot be accurately predicted at the time of filing.

Applicants submit that the "phenotype," to which the Examiner refers, ***is dependent upon the gene of interest*** which is operatively linked to a target DNA sequence to which the fusion protein binds. Applicants respectfully point out that the pending claims do not require a phenotype, but rather recite that the transgene be expressed in cells of the transgenic animal at a level sufficient to produce amounts of the fusion protein that can activate transcription of the gene of interest ***at detectable levels.***

Based on the instant specification and methods of making transgenic animals known at the time of filing, Applicants submit that one of ordinary skill in the art would

be able to make and use the claimed non-human transgenic animal such that the gene of interest is expressed at detectable levels, as required by the claims. In Example 3 of the specification, Applicants teach graded transactivation of a luciferase reporter gene operably linked to the *tet* operator using the claimed fusion protein, wherein transcription of the luciferase gene depends upon activation by the TetR fusion protein. As required by the claims, transactivation by the claimed fusion protein results in ***detectable*** transcription of the luciferase reporter gene, measured as luciferase activity. Based on the examples provided in the instant specification, Applicants show that the claimed gene expression system is a predictable system which provides a precise mechanism for controlling expression of a gene of interest in a detectable manner.

The claimed transgenic animal comprises a gene expression system which allows for transcriptional control of a specific gene of interest such that the transcription is detectable. The Examiner has equated detectable expression with a resulting phenotype, which are not necessarily equivalent. For example, in Example 3 of the specification, the gene of interest is a reporter gene, wherein the phenotype is luciferase activity, which also provides a means of detecting gene expression. In the Hammer *et al.* reference previously cited by the Examiner, however, the authors describe transgenic rats which express B27 and β_2-microglubulin transgenes in a study to determine whether the resulting phenotype resembles human disease conditions, specifically inflammatory conditions. Applicants maintain that the resulting phenotype depends on the nature of the gene of interest and is often the subject of the scientist's hypothesis, wherein the resulting phenotype is unknown until evaluated through the scientific process. Applicants provide a gene expression system which can be used to create transgenic animals comprising a predictable gene expression system, which in turn allows one of ordinary skill in the art the opportunity to study the phenotype of the transgenic animal.

In maintaining the position that the art of making a transgenic animal with a predictable phenotype was unpredictable at the time of filing, the Examiner alleges that "[p]ositional effects, where the transgene is influenced by its site of integration in the host chromosome can have major consequences on the expression of the transgene." The Examiner also asserts that "[s]uch problem would affect the level of expression of the

fusion protein in the transgenic animal, thus it is unpredictable whether the claimed transgenic animal would produce enough fusion protein at [a] high enough level that can regulate the transcription of another gene and produces a phenotype."

As the Examiner is aware, the standard regarding predictability in the art refers to "the ability of one skilled in the art to extrapolate the disclosed or known results to the claimed invention. If one skilled in the art can readily anticipate the effect of a change within the subject matter to which the claimed invention pertains, then there is predictability in the art." (see MPEP 2164.03). Applicants submit that one of ordinary skill in the art would recognize that the process of making a transgenic animal may require the screening of an initial litter of transgenic animals in order to identify founders who express a certain detectable level of the gene of interest, who, in turn, are then used to propagate a transgenic line (see specification at pages 15-16).

In support of Applicants' assertion regarding the predictability of making transgenic animals comprising gene expression systems resulting in detectable expression of a gene of interest, Applicants provide the following references which describe transgenic animals comprising the claimed Tet gene expression system, which is representative of the claimed invention in that a transactivator fusion protein is used to control expression of a gene of interest.

1. Bello *et al.* (1998) *Dev.* 125:2193-2202, describes use of the Tet gene expression control system in *Drosophila* (enclosed as Appendix A);

2. Bieschke *et al.* (1998) *Mol Gen Genet* 258:571-579, describes transgenic flies comprising the reverse Tet gene expression system to control transcription of *tet*-operator linked gene *lacZ* (enclosed as Appendix B);

3. Melfi *et al.* (2000) *J. Mol. Biol.* 304(5):753-763, describes use of the Tet gene expression system in sea urchins to control expression of a *sns* fragment (enclosed as Appendix C);

4. Ridgway *et al.* (2000) *Exp. Cell Res.* 256(2):392, describes the effectiveness of the Tet gene expression system in *Xenopus* (enclosed as Appendix D).

The above-mentioned references describe different transgenic animals or animals containing transgenes, including *Drosophila*, *Xenopus*, and sea urchins. Each of these transgenic animals comprises a transgene encoding a transcriptional activator fusion protein which activates expression of an operator-linked gene of interest. These references are representative of transgenic organisms that contain gene expression systems, and demonstrate the predictable, successful use of a transgene comprising a fusion protein which can activate transcription of a gene of interest operatively linked to a target DNA sequence to which the fusion protein binds. Given that transcriptional regulatory systems described in the above-mentioned references can function in *Xenopus, Drosophila,* and sea urchins, all of which are phylogenetically diverse organisms, it would be expected that the transcriptional regulatory system of the claimed invention would also function in diverse organisms. If the Examiner maintains the assertion that making transgenic animals in accordance with the claimed invention is unpredictable, Applicants respectfully request that the Examiner point to evidence to support this assertion and/or explain why each of the submitted references does not mention the predictability of creating a transgenic animal expressing a gene of interest at detectable levels.

The Examiner maintains that previously cited Hammer *et al.* teaches that "a phenotype produced in one species is not predictive for the same phenotype in another species." The Examiner has acknowledged that Hammer *et al.* is not indicative of the state of the art at the time of filing due to its earlier publication date, yet has maintained the rejection in view of this reference and requests that Applicants provide evidence "that such unpredictability is resolved at the time of filing." Applicants respectfully point out that the claims do not specify a phenotype, but rather require that the claimed fusion protein activate transcription of the gene of interest at detectable levels. Applicants also respectfully maintain that at the priority date of the instant application, the teachings set forth in the specification with regard to the general construction of transgenic organisms (see *e.g.*, pages 17-19), were routinely utilized in the production of a variety of transgenic organisms, (see, *e.g.*, the above-referenced scientific articles and those references submitted as Appendices A to G in the response filed October 15, 2003).

Moreover, as the Examiner is well aware, the fact that some experimentation may be necessary to produce a transgenic non-human animal with a gene at a specific location, does not constitute a lack of enablement as long as the amount of experimentation is not unduly extensive. *Amgen Inc. v. Chugai Pharmaceutical Co., Ltd.*, 927 F.2d 1200, 1213 (CAFC 1991). A considerable amount of experimentation is permissible if it is merely routine, or if the specification provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands* 8 USPQ2d 1400-1407, 1404 (CAFC, 1988).

The Examiner further asserts that "one skilled in the art would not know how to use a transgenic animal with claimed genotype, but shows no phenotypic feature." In contrast, Applicants respectfully submit that one of ordinary skill in the art would unquestionably recognize the advantage of the claimed invention, in that it provides transgenic animals comprising a gene expression system with the ability to control transcription through graded transactivation (see page 2, lines 15-19 of instant specification). As evidence that one of ordinary skill in the art would know how to use the claimed transgenic animal with the claimed genotype, Applicants refer the Examiner to the above-referenced scientific articles which describe transgenic animals comprising a similar gene expression system, as well as the above-stated arguments regarding phenotype. Applicants also provide the following references which describe transgenic animals, *e.g.*, mice, comprising the claimed genotype.

Angeletti *et al.* (2003) *Inv Optham Vis Sci* 44:755 (enclosed herewith as Appendix E) describes an improved system for gene expression in retinal cells using an activator transgene like that claimed. The authors of the Angeletti reference use the tTA1 activator for cell-specific and time-controlled transcription of the luciferase reporter gene. As described in the instant specification at page 34, table 1, the tTA1 transactivator comprises four copies of the mutated acidic region of VP16 set forth as SEQ ID NO: 1, *i.e.,* TetR-F. The tTA1 transactivator described in the Angeletti reference is identical to that described in Table 1 of the specification and described in the pending claims.

Shigehara *et al.* (2003) *J Am Soc Nephroly* 14: 1998 (enclosed herewith

as Appendix F) describes transgenic mice comprising the claimed transactivator in a study of successful kidney-specific transgene expression. As described at page 1999 in the first paragraph of the Materials and Methods section, the authors of the Shigehara reference used a transactivator comprising the "FFF minimal activator domains" to activate expression of reporter gene β-gal and podocyte marker WT-1. The transactivator domain used in the transgenic animals described in the Shigehara reference is the same as that claimed by Applicants and is described in Table 1 of the instant specification.

In addition, the following reference all describe transgenic mice with tissue specific transgene expression using a transactivator comprising tTA2, like that described in Table 1 of the instant specification. Hasan *et al.* (2001) *Genesis* 29:116 (enclosed herewith as Appendix G) describes transgenic mice wherein the transactivator of the instant invention controls expression of the firefly luciferase gene in a study of noninvasive ways of imaging living mice. Gallagher *et al.* (2003) *J Am Soc Nephroly* 14: 2042 (enclosed herewith as Appendix H) describes kidney-specific expression of a transgene. Finally, Pot *et al.* (2002) *J Cell Biol* 159: 29 (enclosed herewith as Appendix I) describes transgenic mice with *nogo A* expression in Schwann cells.

Based on the above-mentioned references, Applicants submit that in contrast to the Examiner's assertion, one of ordinary skill in the art would appreciate the advantages provided by the claimed invention in controlling gene expression in transgenic animals.

In view of the teachings in the specification and the general knowledge in the art, the specification has provided sufficient guidance to the ordinarily skilled artisan for making and using the invention without undue experimentation. Accordingly, the specification meets the enablement requirement and Applicants respectfully request that the rejection of claims 32 and 40-54 under U.S.C. § 112 first paragraph, be withdrawn.

The Examiner has also rejected claim 51 under U.S.C. § 112 first paragraph for encompassing homologous recombinant non-human transgenic animals other than mice. The Examiner alleges that only mouse embryonic stem (ES) cells were available at the time of filing. Applicants respectfully submit that the use of homologous recombination for site specific transgene integration had been successfully demonstrated in a number of organisms at the time instant application's priority date. In addition, the specification

describes how to prepare transgenic organisms by homologous recombination such that the transgene is integrated at a predetermined location in the genome. Specific, detailed guidance is provided regarding the vectors required for homologous recombination (see e.g., pages 17-18 of specification). For example, in a preferred embodiment, the vectors contain the DNA encoding the fusion protein flanked at its 5' and 3' ends with additional nucleic acids corresponding to the eukaryotic gene at which homologous recombination is to occur, and can readily be prepared using standard molecular biology techniques known to those skilled in the art. Additionally, the specification cites, and incorporates by reference, several references describing homologous recombination methodologies (see *e.g.*, page 18, lines 12-17). In the interest of expediting prosecution, however, Applicants have cancelled claims 51-54. Thus, the rejection of claims 51-54 is rendered moot.

Rejection of Claim 51 Under 35 U.S.C. 112, Second Paragraph

Claim 51 is rejected under 35 U.S.C. § 112, first paragraph as being indefinite to particularly point out the claimed invention. Specifically, the Examiner states use of the term "homologous recombinant non-human transgenic animal" makes the claims indefinite "because it is unclear what the term encompasses." Applicants respectfully traverse this rejection. In contrast to the Examiner's assertion, the term "homologous recombinant animal" is a term of art which indicates an animal which has been modified by homologous recombination between a gene and a DNA molecule introduced into the cell of the animal, as described in the specification at page 16, lines 19-21. In the interest of expediting prosecution, however, Applicants have cancelled claim 51.

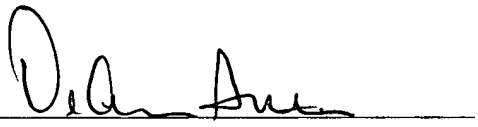
Double Patenting Rejection of Claims 52-54

Claims 52-54 have been rejected under CFR 1.75 as being the substantially the same as pending claims 40-42. Claims 52-54 have been cancelled, thus rendering the double patenting rejection moot.

CONCLUSION

If a telephone conversation with Applicants' Attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call Applicants' Attorney at (617) 227-7400.

Respectfully submitted,



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Spatial and temporal targeting of gene expression in *Drosophila* by means of a tetracycline-dependent transactivator system

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SUMMARY

In order to evaluate the efficiency of the tetracycline-regulated gene expression system in *Drosophila*, we have generated transgenic lines expressing a tetracycline-controlled transactivator protein (*tTA*), with specific expression patterns during embryonic and larval development. These lines were used to direct expression of a *tTA*-responsive promoter fused to the coding region of either the β-galactosidase or the homeotic protein Antennapedia (ANTP), under various conditions of tetracycline treatment. We found that expression of β-galactosidase can be efficiently inhibited in embryos and larvae with tetracycline provided in the food, and that a simple removal of the larvae from tetracycline exposure results in the induction of the enzyme in a time- and concentration-dependent manner. Similar treatments can be used to prevent the lethality associated with the ectopic expression of ANTP in embryos and, subsequently, to control the timing of expression of the homeoprotein ANTP specifically in the antennal imaginal disc.

Our results show that the expression of a gene placed under the control of a tetracycline-responsive promoter can be tightly controlled, both spatially by the regulatory sequences driving the expression of *tTA* and temporally by tetracycline. This provides the basis of a versatile binary system for controlling gene expression in *Drosophila*, with an additional level of regulation as compared to the general method using the yeast transcription factor GAL4.

Key words: Tetracycline, Gene expression, *Drosophila*, *Antennapedia*

INTRODUCTION

An essential and general experimental approach to analyse the function of a gene in a whole organism is to examine the phenotypic consequences of its directed expression in certain cells, or at a developmental stage, in which the gene is normally silent. In *Drosophila*, two major systems have been designed to achieve the conditional expression of gene constructs integrated into the genome. In the first, the coding sequence of the gene of interest is placed under the control of promoters inducible according to the culture conditions. The *hsp70* gene promoter is commonly use for that purpose (see Schneuwly et al., 1987 and references therein). High levels of induction can be obtained at well-defined time periods during development upon exposure of the organism to elevated temperatures. This advantage is often limited because ectopic expression occurs in all cells and endogenous genes are repressed during heat shocks. Consequently, side defects including lethality may mask the result of the ectopic expression in the desired cell type(s). It is also frequently necessary to repeat heat shocks over an extended time period to observe the phenotypic consequences, and it may be difficult to distinguish between the primary and secondary effects of the overexpression (e.g. Gibson and Gehring, 1988).

An alternative approach is to engineer a gene construct inducible by a single transcription factor whose activity can be controlled *in vivo*. The ability of the yeast transcription factor GAL4 to activate transcription in *Drosophila* (Fisher et al., 1988) has been exploited to generate a versatile method for targeting gene expression in this organism (Brand and Perrimon, 1993). Any gene of interest can be placed under the control of a promoter containing GAL4 upstream activating sequences (UAS) and integrated stably into the genome of a parental line (responder strain), since it remains silent in the absence of GAL4. Tissue-specific expression of the gene is obtained upon crossing to a second parental line (driver strain) expressing the transcription factor under the control of a suitable promoter. Although a large number of strains expressing GAL4 in a wide variety of patterns can be selected on the basis of the expression of a reporter gene bearing the UAS sequences (Brand and Perrimon, 1993; Yeh et al., 1995; Calleja et al., 1996), only a few of them have been used to direct expression of functional proteins in the post-embryonic stages of development (Brand and Perrimon, 1993; Capdevila and Guerrero, 1994; Hinz et al., 1994; Speicher et al., 1994; Rimmington et al., 1994; Ferver et al., 1995; Halder et al., 1995; Zink and Paro, 1995; Freeman, 1996; Morimura et al., 1996). The limit in this binary system lies in the lack of

temporal control, which remains primarily determined by the regulatory sequences driving GAL4. Because most of the gene-specific enhancers are active at various stages of development in *Drosophila*, GAL4-mediated induction is frequently observed from the embryonic stage onwards, and often results in premature lethality (e.g. Halder et al., 1995). To overcome this difficulty, Flp-mediated recombination has been used to achieve conditional expression of a transgene upon recombination of an FRT cassette separating the coding region from a promoter (Struhl and Basler, 1993). This approach can be used to control the expression of GAL4 (Pignoni and Zipursky, 1997). In any case it requires the combination of various specific constructs and it can be applied only to tissues in which cell division occurs, since it results in the generation of clones of expressing cells.

Extension of the more versatile binary system for post-embryonic studies could be achieved with the use of a regulatory protein modulated in the organism with an innocuous effector. One of the best candidates is the tetracycline-dependent transactivator (*tTA*) comprising the tetracycline repressor of *E. coli* (*tetR*) and the strong transcriptional activation domain of the herpes simplex virus protein VP16 (Gossen and Bujard, 1992). The high affinity and specific binding of *tetR* to the tetracycline operator sequences (*tetO*) can be inhibited by tetracycline (Hillen and Wissmann, 1989) and is thought to result from a conformational change of *tetR* upon association with tetracycline (Hinrichs et al., 1994). In HeLa cells, *tTA* was found to stimulate transcription of a promoter bearing a multimerized *tetO* by several orders of magnitude, and a fast and reversible switch of the *tTA*-dependent promoter was obtained upon addition or removal of tetracycline from the culture medium (Gossen and Bujard, 1992). These features have been extensively exploited in tissue culture where tetracycline levels can be tightly controlled. Tetracycline-regulated expression of reporter genes was also demonstrated in whole plants (Weinmann et al., 1994) and in transgenic mice (Hennighausen et al., 1995; Kistner et al., 1996). In the latter case, the efficiency of tetracycline, administrated by slow-release tetracycline pellets or in drinking water, has been mostly evaluated by analysing the expression of sensitive reporter genes, although a few examples of successful expression of proteins have been reported (see Shockett and Schatz, 1996 for a review).

In this study we determined the functional properties of *tTA* in *Drosophila* by expressing this regulatory protein under the control of various promoters. Using a *lacZ* reporter gene placed under the control of a promoter bearing seven copies of *tetO*, we found that expression of β-galactosidase can be tightly controlled in embryos and larval tissues. Furthermore, we used *tTA*-expressing strains and tetracycline treatments to drive tissue-specific ectopic expression of the homeoprotein *Antennapedia (Antp)* at different stages of development. Our results demonstrate the usefulness of the *tet* system in *Drosophila*.

MATERIALS AND METHODS

DNA constructs

Most of the constructs were assembled by multiple step cloning according to standard methods (Sambrook et al., 1989). Further details and maps are available upon request.

tTA driver constructs

hsp70-tTA

The *tTA* coding region was isolated as a 1.1 kb *Eco*RI-*Bam*HI fragment from pUHD 15-1 (Gossen and Bujard, 1992) and cloned into CaSpeR-hs (Thummel and Pirrotta, 1992)

RHT (*rosy*, *hsp70* promoter, *tTA*)

This P element vector derives from the enhancer-test vector HZ50PL (Hiromi et al., 1985). The *tTA* coding region is flanked by the minimal promoter and the poly(A) sequences of *hsp70* (Fig. 1A).

ey-tTA and *HoxA7-tTA*

The *eyeless (ey)* gene enhancer (a 3.5 kb *Kpn*I fragment from *ey* Eco 3.6) (B. Hanck, T. Eggert, W. J. Gehring and U. Walldorf, unpublished) and a 630 bp fragment of the intron of the *HoxA7* gene from pB6 (Haerry and Gehring, 1996) were cloned in RHT to give *ey-tTA* and *HoxA7-tTA*, respectively.

Tetracycline-responder constructs

tetO-lacZ

The heptameric repeat of the tet operator was isolated as a 310 bp *Eco*RI-*Kpn*I fragment from pUHC 13-3 (Gossen and Bujard, 1992) and cloned upstream of the P-*lacZ* fusion of the enhancer-test vector CPLZ (Wharton and Crews, 1993). CPLZ contains the P-element transposase promoter (up to –42 from the cap site) and the N-terminal transposase sequence fused in-frame with *lacZ* and the polyadenylation signal of *hsp70*.

WTP (white-*tetO*-P promoter)

This P-element vector was constructed to express any gene under the control of a tetracycline-responsive promoter. It contains the vector backbone of CPLZ, the heptameric repeat of the tet operator, the P-element promoter and leader sequences from Carnegie 4 (Rubin and Spradling, 1983) and the polyadenylation signal of SV40.

tetO-Antp and *tetO-AntpΔHD*

The cDNAs encoding a full-length ANTP protein or a variant with a deletion of the homeodomain were isolated as *Not*I fragments from pHSSΔA and pNHT-A11, respectively (Gibson et al., 1990) and cloned into the corresponding site of WTP.

Germline transformation and *Drosophila* strains

P-element mediated transformation of ry^{506} or *y ac* w^{1118} recipient strains was carried out essentially as described (Spradling, 1986). A description of the markers and balancer chromosomes indicated in Figs 3 and 4 can be found in Lindsley and Zimm (1996). A405.1 M2 and rK781 have been described (Wagner-Bernholz et al., 1991; Flister, 1991).

Tetracycline media and treatments

A tetracycline-containing medium suitable for larval feeding and maintenance of adults was obtained by mixing 100 ml of tetracycline solution (tetracycline hydrochloride (Sigma) diluted with sterile water at the required concentration), 25 g of Instant *Drosophila* Food (Carolina Biological Supply) and 1 g of dry yeast.

Tetracycline was provided to adult females by placing 50-70 virgins in a glass vial containing a 2.5 cm filter (Whatman grade 3 MM) soaked with 500 μl of a 4% sucrose solution with tetracycline at the appropriate dilution. Females were fed for 3-4 days, following a daily cycle of 16 hours on tetracycline-containing filters and 4 hours on standard food supplemented with a drop of yeast paste. Males of the relevant genotype were placed with females on tetracycline and allowed to mate overnight before the beginning of egg collections. For

experiments requiring larval feeding, batches of 100-200 eggs harvested from grape juice plates were placed on pieces of nylon mesh, and allowed to develop at 25°C on tetracycline-containing food. When necessary, larvae were separated from their food by floating in 30% glycerol, collected with forceps, washed with PBS and transferred on standard food in groups of 50 to 100.

Phenotypic analyses

Embryos and larval tissues were fixed and stained for β-galactosidase as described (Bellen et al., 1989). Adult heads were separated from the body of narcotised flies and holes were made into the cuticle to facilitate penetration of the fixative. For antibody staining of embryos and examination of cuticular phenotypes, standard procedures were applied (Ashburner, 1989).

RESULTS

We have designed a general system to express *tTA* under the control of regulatory sequences (RHT driver construct), in order to direct the expression of a gene of interest under the regulation of a tetracycline-responsive promoter (WTP responder construct). The gene constructs can be stably propagated into the genome of separate strains, and *tTA*-dependent gene induction is obtained in the F_1 offspring of the cross where it can be controlled by tetracycline (Fig. 1A).

tTA is a potent transactivator in *Drosophila*

In order to analyse the transactivation potential of *tTA*, we have used an indirect heat shock assay to drive ubiquitous expression of *Antp* in embryos. Heat shock assays were performed on embryos carrying *tTA* under the control of the *hsp70* gene promoter (*hsp70-tTA*) and a WTP derivative carrying a full-length *Antp* cDNA (*tetO-Antp*). Independent transformants were found to give an identical embryonic phenotype to the H4 line that carries a direct *hsp70-Antp* construct (Fig. 1B; see also Gibson and Gehring, 1988 for a complete description of the H4 line). In contrast, heat-shocked embryos carrying a WTP derivative with a deletion of the homeodomain (*tetO-Antp*ΔHD) or the empty WTP vector (*tetO–*) showed a wild-type cuticle and developed to the adult stage (Fig. 1B and data not shown). A western blot of embryonic extracts prepared from heat-shocked embryos and probed with an ANTP-specific monoclonal antibody (Condie et al., 1991) reveals similar levels of ANTP expression (Fig. 1C). In addition, transformation of the adult antenna into a mesothoracic leg can be obtained when heat shocks are applied to third instar larvae (Gibson and Gehring, 1988; D. Resendez-Perez, B. Bello and W. J. Gehring, unpublished). Taken together, these results show that *tTA* can activate transcription of a promoter that contains *tetO* sequences without any toxic effect of this regulatory protein in *Drosophila*.

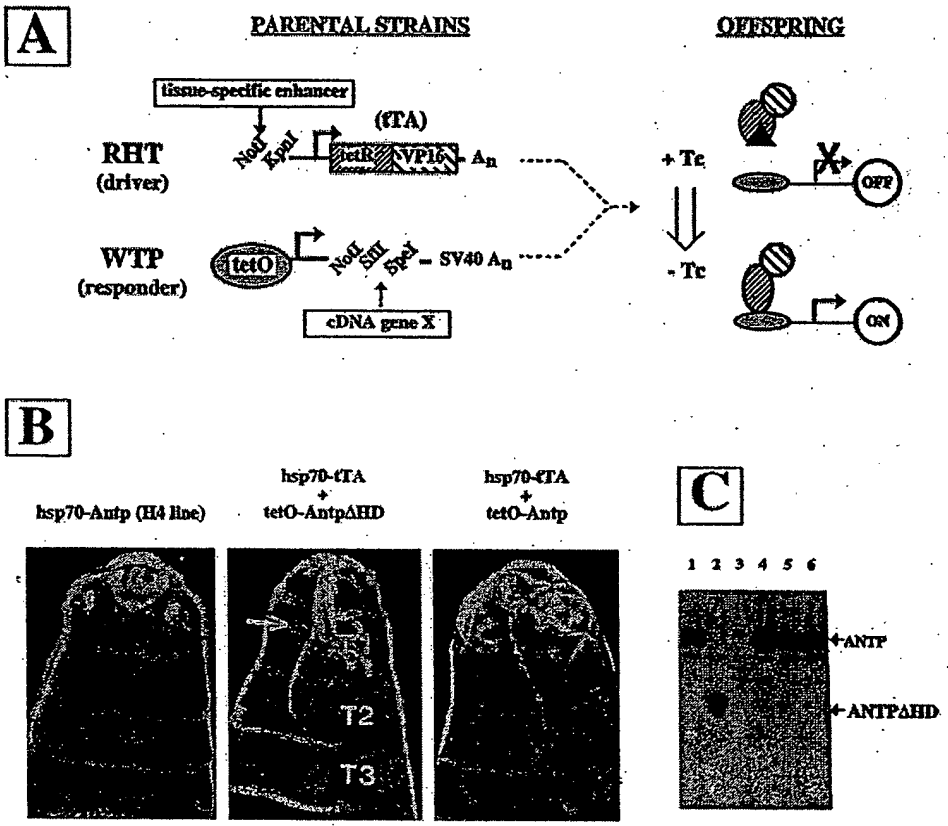

Fig. 1. Transgenic constructs and the transactivation potential of *tTA* in *Drosophila*. (A) Schematic representation of the tetracycline-inducible system in *Drosophila*. The transformation vectors RHT and WTP are, respectively, designed to express *tTA* under the control of enhancer-like elements and any cDNA under the regulation of a *tTA*-responsive promoter that contains *tetO* sequences. Derived constructs are integrated and propagated in two separate parental strains. In absence of tetracycline (–Tc), the binding of *tTA* results in the activation of gene X with the spatial and temporal specificities determined by the driver construct (on state). Raising the offspring on a tetracycline-containing medium is used to prevent the binding of *tTA* in order to keep the responder construct silent up to a certain point of time (off state). Stopping tetracycline exposure allows time-specific induction of gene X upon withdrawal of the antibiotic. (B) Cuticular preparations of embryos heat shocked for 30 minutes at 6.5 hours of embryogenesis. All embryos carry a single copy of the constructs indicated at the top. Note the similar defect in head evolution in *hsp70-Antp* and *hsp70-tTA*; *tetO-Antp*, and the T1 to T2 transformation when compared to the wild-type cuticle of *hsp70-tTA*; *tetO-Antp*ΔHD embryos. The identical segmental transformation is revealed by the disappearance of the characteristic denticle belt of the first thoracic segment (white arrow), indicating a transformation of T1 towards T2. The head segments are also transformed towards T2. T1, T2, T3: first, second and third thoracic segments respectively. (C) Detection of the ANTP proteins by western blot analysis in crude extracts of embryos heat shocked for 2 hours with a 4 hour recovery. Lane 1, *hsp70-Antp* embryos (H4 line); lanes 2- 6, embryos carrying *hsp70-tTA* and either *tetO-Antp* ΔHD (lane 2) or the empty WTP vector (lane 3), or independent insertions of *tetO-Antp* (lanes 4-6).

Tetracycline-controlled expression of *lacZ* during larval development

In order to express *tTA* during larval development, we inserted the eye-specific enhancer of the *ey* gene (Quiring et al., 1994) into the vector RHT and generated *ey-tTA* transformants. The expression of *tTA* was detected specifically in the eye imaginal disc by means of a *lacZ* reporter gene placed under the control of a promoter bearing *tetO* sequences (Fig. 2). Detection of β-galactosidase activity in the eye disc of the larvae reveals two essential features. First, enzymatic activity is detectable within less than 15 minutes of incubation with X-gal revealing a high level of expression of *tTA*. Second, this activity is detected in the eye disc over an extended period of development with a dynamic pattern (Fig. 2A, top row). In the early third instar, expression is detected uniformly in the eye part of the eye-antennal disc (left panel), which corresponds to the undifferentiated cells of the eye epithelium (Ready et al., 1976). The same pattern was observed in second instar larvae (not shown). Eye discs stained at different times during the third instar show that β-galactosidase activity gradually fades in the anterior region of the disc as the morphogenetic furrow moves anteriorly.

To determine whether tetracycline incorporated in the larval food could inactivate *tTA*, we stained early third instar larvae raised on media containing increasing concentrations of the antibiotic (Fig. 2A, time 0). No activity could be detected in the eye-disc of larvae exposed to as little as 0.1 μg/ml even after prolonged staining (24 hours) in X-gal solution (Fig. 2A, left column). Lower concentrations failed to inactivate lacZ expression (not shown). The results indicate an efficient uptake of tetracycline from the food and its diffusion to the imaginal discs, leading to a complete inactivation of *tTA* in a concentration-dependent manner. The same dose-response was obtained for larvae exposed to tetracycline for approximately 2 more days, suggesting that the concentration of tetracycline in the larval haemolymph does not change dramatically during the feeding period (not shown). In contrast, stopping the larval exposure to tetracycline was expected to decrease the level in the haemolymph and to restore the ability of *tTA* to bind and transactivate the *lacZ* reporter gene. To test this hypothesis, early third instar larvae were transferred to standard food, fixed and stained every 6 hours for β-gal activity (Fig. 2A). Enzymatic activity was detected 6 hours after the removal of the larvae from the medium containing 0.1 μg/ml tetracycline (Fig. 2A, second row from the top) or in a range of 18 to 24 hours when the larvae were exposed to 1 μg/ml or 10 μg/ml tetracycline, respectively (Fig. 2A, third and fourth row from the top). After induction, 12-24 hours were necessary to obtain

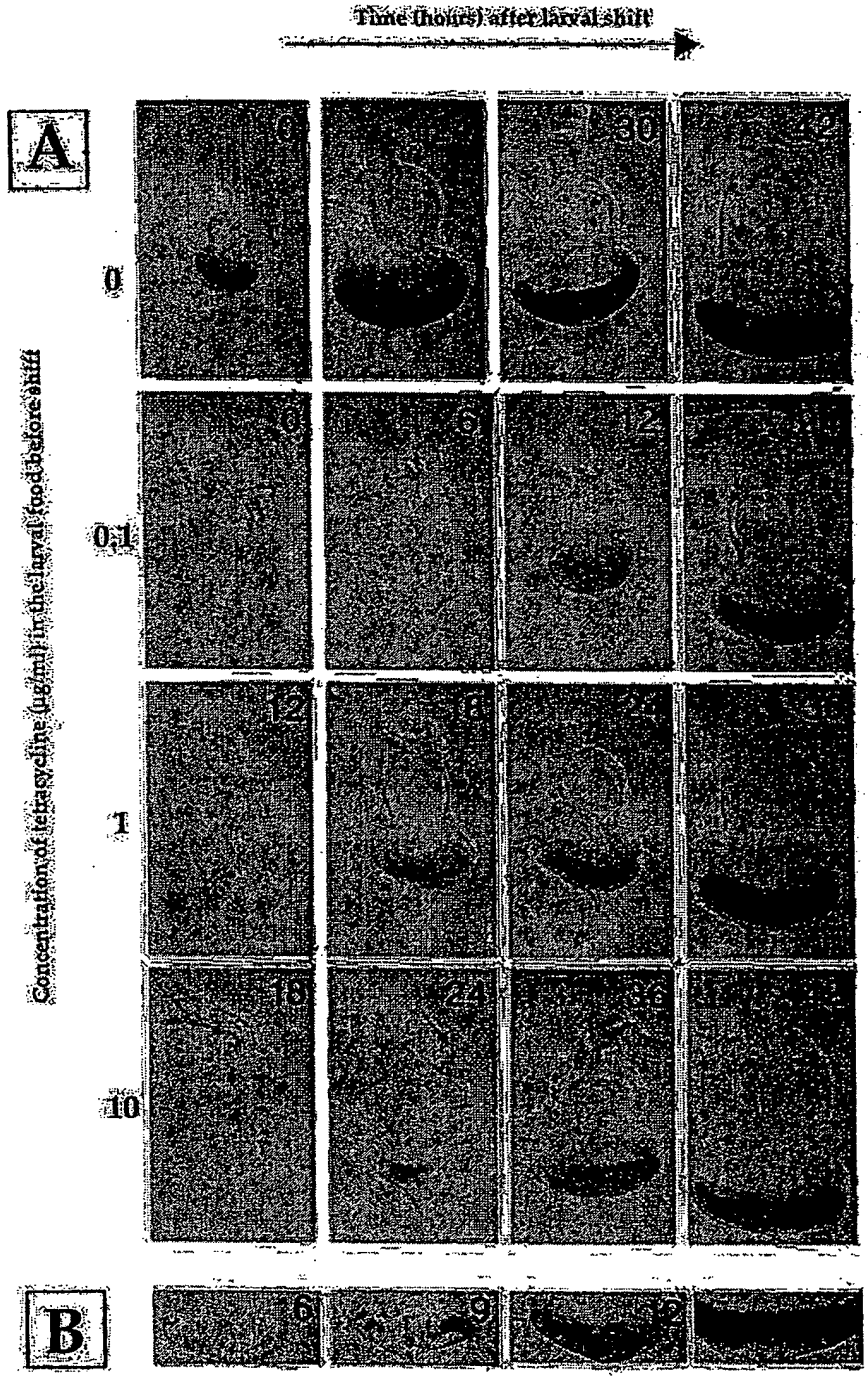

Fig. 2. Tetracycline controlled expression of *lacZ* in the eye imaginal disc driven by the *ey-tTA* strain. (A) Larvae of the genotype *ey-tTA/+*; *tetO-lacZ/+* were raised in the presence of increasing concentration of tetracycline, shifted to standard food at the early third instar and dissected at different times after shifting, indicated in hours at the top right of every figure. (B) Temporal profile of *lacZ* induction from larvae removed from 0.1 μg/ml tetracycline during the late third instar. Note the increasing number of cells expressing *lacZ*.

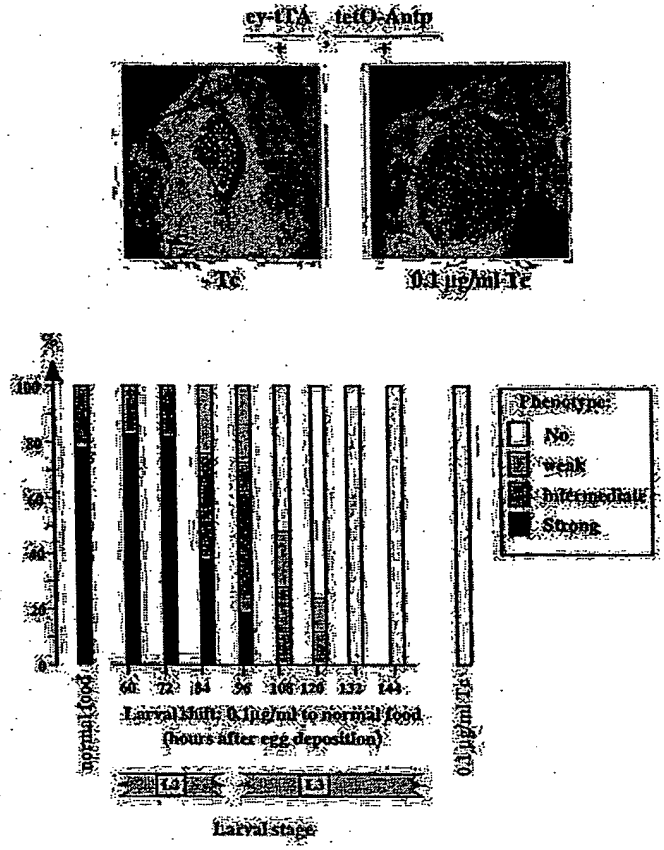

Fig. 3. Induction of *Antennapedia* in the eye imaginal disc by means of tetracycline. (Top) Eyes of adult flies raised in absence (–Tc) or in the presence of 0.1 µg/ml tetracycline (Tc) in the larval food. (Bottom) Semi-quantification of the eye phenotype in adults derived from larvae raised with 0.1 µg/ml tetracycline and shifted to standard food at 12 hour intervals. At least 50 adults were scored at every time point. Larval stages were identified on the basis of the morphology of the tracheal system and the anterior spiracles.

a comparable staining intensity in imaginal discs isolated from larvae exposed to tetracycline and control larvae. These data show that the withdrawal of the larvae from tetracycline is followed by a rapid induction of the *tetO-lacZ* transgene under the control of *tTA*. The concentration- and time-dependent expression of *lacZ* is likely to reflect the need to lower the concentration of tetracycline in the disc cells below a certain threshold level, which allows *tTA* to bind the tet operator and to stimulate transcription of the promoter. A careful examination of the staining patterns also suggested that the *tetO-lacZ* transgene was not turned on in every cell at the same time after withdrawal of tetracycline (Fig. 2A). To confirm this observation, we performed larval shifts during the second half of the third instar, when expression of *tTA* is uniform in the posterior part of the eye disc. Upon removal of the larvae from 0.1 µg/ml tetracycline, induction of *lacZ* can clearly be observed in a gradually increasing number of cells (Fig. 2B). Similar observations were obtained with different *tTA*-expressing strains, suggesting variations in the kinetics of the clearance of the antibiotic and/or the trancriptional activation.

Tetracycline-controlled expression of *Antennapedia* targeted to the eye disc

To determine the relevance of the data obtained with the *lacZ* reporter gene we used the same driver strain to direct expression of *Antp* under various conditions of tetracycline treatment. In the absence of tetracycline, adults obtained from

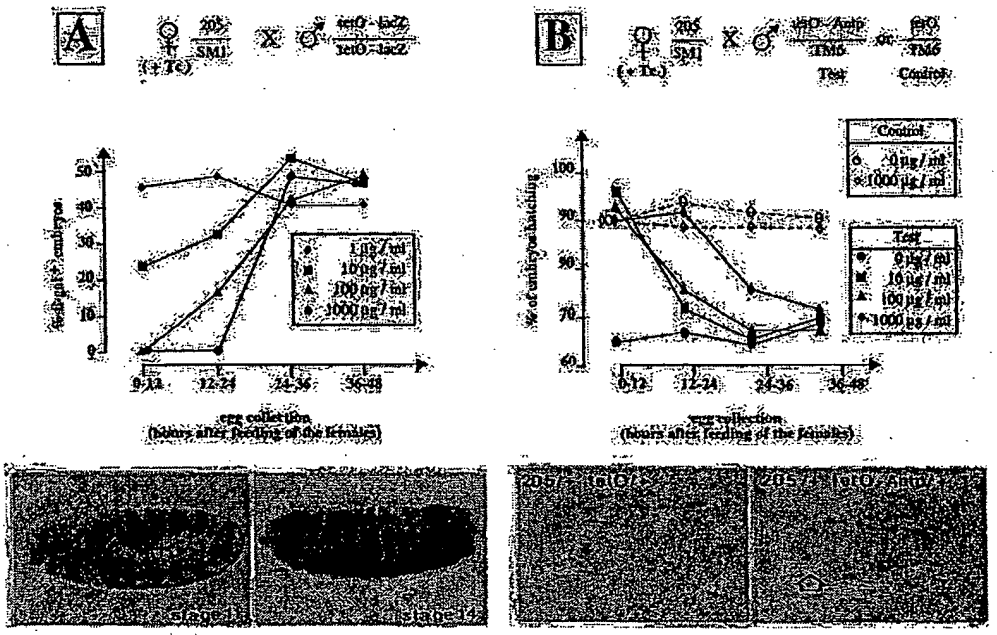

Fig. 4. Inhibition of *tTA*-dependent gene expression in embryos by maternal transmission of tetracycline. (A) Repression of the *lacZ* reporter driven by the 205 strain, shown at the bottom by immunodetection of β-galactosidase. Embryos collected over successive 12 hour periods following the feeding of 205/SM1 females with tetracycline, were stained for β-gal activity. At least 100 embryos older than the germ band extended stage (stage 11) were scored at every time point. 50% of transheterozygotes were expected in the offspring of the cross indicated at the top. (B) Tetracycline-dependent rescue of embryonic lethality by repression of the *tetO-Antp* transgene. Embryos collected over 12 hour periods after feeding of their mothers with tetracycline were allowed to develop at 25°C and the percentage of hatching larvae determined. At least 200 embryos were scored at every time point. 25% of transheterozygotes are expected from the cross indicated at the top. *tetO* refers to an insertion of the empty vector WTP on the third chromosome, used as a control. Bottom: cuticular phenotype of embryos of the indicated genotype. Note the failure of head involution marked by the open arrow in embryos expressing *Antp* under the control of the 205 strain.

a cross between the parental *ey-tTA* and *tetO-Antp* strains showed reduced and irregular compound eyes (Fig. 3, –Tc). Since *Antp* is normally not expressed in the eye disc (Wirz et al., 1986), its expression in this tissue would interfere by unknown mechanisms with the normal development of the eye. No other morphological defects could be detected in adults, in agreement with the eye-specific expression of *tTA* detected with the *lacZ* reporter. To ascertain that the eye phenotype resulted from the ectopic expression of *Antp* in the eye disc, we raised larvae on a medium containing tetracycline at various concentrations. The eyes were restored to a wild-type appearance with 0.1 μg/ml tetracycline (Fig. 3, top) but not with 0.01 μg/ml tetracycline, in good agreement with the dose-dependent repression of the *lacZ* reporter (Fig. 2A).

As indicated with the *lacZ* reporter, the directed expression of *Antp* by the *ey-tTA* strain should occur continuously throughout larval development and shift rapidly during the third instar when the cells undergo differentiation. We used tetracycline to control the timing of *Antp* overexpression in order to determine the functional significance of this dynamic pattern of expression, with respect to the alteration of the eye development. Newly hatched larvae were first fed with 0.1 μg/ml to inhibit *tTA* activity and then transferred to a standard medium every 12 hours to induce *Antp*. Adults were scored for eye defects and classified according to an arbitrary scale of strong, intermediate, weak or no detectable eye phenotype (Fig. 3, bottom). All the adults derived from larvae exposed to tetracycline up to the second instar showed strong eye defects in a range indistinguishable from their siblings raised in absence of tetracycline. In contrast, subsequent shifts allowing induction of *Antp* from the early third instar onward led rapidly to a complete rescue of the eye morphology. These results confirm the efficiency of the tetracycline control with a functional homeoprotein and suggest that the alteration in the normal eye development is mostly dependent on the ectopic expression of *Antp* in the undifferentiated cells of the eye epithelium.

Repression of *tTA*-dependent gene expression in embryos by maternal transmission of tetracycline

The embryonic development of *Drosophila* is not easily amenable to antibiotic treatment since the egg is protected by an impermeable set of eggshells but it is relatively fast (22-24 hours at 25°C) and maternal components are transmitted to the oocyte by the nurse cells and the follicle cells in the female ovaries. The influence of tetracycline given to the parental females was first tested on the strong *lacZ* expression driven by the *tTA* construct of the line 205 (Fig. 4A). This driver line 205 was isolated among twelve independent transformants of the *HoxA7-tTA* construct (see Materials and Methods) because of its unique expression pattern observed in the antennal disc (Fig. 5), the leg discs, the central nervous system, the epidermis and various internal tissues (not shown). Since the other lines showed a reproducible pattern in the eye disc and the larval brain due to the *HoxA7* enhancer (not shown), the line 205 is likely to reflect a modified expression of the transgene under the influence of genomic regulatory sequences flanking the integration site (Wilson et al., 1990). When assayed in embryos with the *lacZ* reporter gene, expression of *tTA* in the 205 strain starts at the end of germ band extension, about 5 hours after egg laying (AEL), and is detected mostly in the trunk region with a segmentally repeated pattern (Fig. 4A, bottom). This pattern changes rapidly, so that at the end of germ band retraction (approximately 10 hours AEL), strong expression is detected all over the ectoderm. The staining appears patchy in the cephalic segments and is not uniformly distributed in the thoracic and abdominal segments (Fig. 4A, bottom). After treatment of the females with tetracycline (see Materials and Methods), repression of the *lacZ* reporter is mostly effective in the eggs collected immediately after the end of exposure to the antibiotic and is dose-dependent (Fig. 4A, top). Repression was obtained in 100% of the eggs collected within 12 hours after the treatment of the females with 100 μg/ml or more of the antibiotic, and in a large fraction of them with 10 μg/ml. The gradual loss of repression observed in the eggs collected later is likely to reflect a decrease of the maternal pool of tetracycline accompanying the continuous production of eggs.

The same procedure of tetracycline treatments was tested for its effect on the survival rate of embryos carrying the driver construct 205 and either a *tetO-Antp*, or an empty responder construct (*tetO–*), as control (Fig. 4B). Examination of the embryonic cuticles revealed major defects in the formation of the head (Fig. 4B, bottom), a phenotype reminiscent of heat-shocked embryos in which *Antp* was ubiquitously expressed (Fig. 1B), although no homeotic segmental transformations were observed. This might reflect a different level of induction of *Antp* as compared to the use of a heat shock promoter but it is more likely to be due to a difference in the timing and the spatial expression of the homeoprotein, since transformations obtained by heat shocks are optimal when induced at 5-7 hours of development (Fig. 1B, see also Gibson and Gehring, 1988), at a stage when the 205 driver is mostly active in the trunk and is not ubiquitously expressed (Fig. 4A, bottom). Nevertheless, line 205 allowed us to test the effect of tetracycline on the survival rate of the embryos and, as shown in Fig. 4B, the embryonic lethality could be overcome in a dose-dependent manner by providing tetracycline to the females. The embryonic rescue was in good agreement with the tetracycline-mediated repression of the *tetO-lacZ* transgene (Fig. 4). These two independent assays clearly demonstrate the possibility to inactivate *tTA* in embryos in order to keep a promoter silent during this stage of development.

Targeted mis-expression of *Antp* in larvae following embryonic rescue

The efficient inactivation of *tTA* in embryos prompted us to analyse the fate of tetracycline-rescued embryos in more detail. As expected, embryos did not develop to the adult stage in the absence of tetracycline in the larval food, whereas addition of tetracycline led to the recovery of viable adults in a concentration-dependent manner (the quantitative data are available upon request). In addition, larvae raised under optimal conditions were shifted to standard food at various times to allow induction of *Antp*. Shifts performed during the late third instar led to the recovery of pharates or adults, whereas larvae shifted earlier essentially failed to undergo metamorphosis. Examination of the adults revealed very specific morphological modifications of the antennae and the head vertex (Fig. 5K-M), in the area expressing the *lacZ* reporter under the regulation of the 205 driver line (Fig. 5E-G). In contrast, transheterozygotes raised continuously with 10 μg/ml tetracycline showed wild-type structures (Fig. 5H-J),

demonstrating the highly specific alterations in the development of adult flies following the mis-directed expression of *Antp* by the 205 line. As revealed with the *lacZ* reporter, expression of *tTA* follows a dynamic spatial pattern in the primordia of the antenna from the mid-third instar onwards (Fig. 5A-D) and in the presumptive area of the occelli from the larval/pupal transition onward (Fig. 5C,D). Moreover, the lag in induction imposed by the removal of the larvae from tetracycline exposure suggests that the alterations of adult structures mostly result from the ectopic expression of *Antp* during the pupal stage. No defects were found in the legs or the palps where the 205 driver is also strongly expressed (not shown), in agreement with previous observations showing that only the derivatives of the eye-antennal disc respond to the ubiquitous expression of *Antp* induced by heat shock (Gibson and Gehring, 1988). These latter studies showed that repeated pulses of heat-shock expression are required during the third larval instar to achieve complete antenna to leg transformations. Our results confirm previous observations showing that the late larval induction of *Antp* does not induce fully differentiated morphological markers of the leg (Scanga et al., 1995; Larsen et al., 1996). Although we observe different arrangements of bristles on the antenna, none of them showed the bracts characteristic for leg bristles.

Directed expression of *Antp* in the antennal disc by *tTA* activates *rK781*

Since the observation of adult phenotype required late larval shifts, we asked whether the consequences of *tTA*-dependent expression of *Antp* could be directly assayed in the imaginal discs. As a marker, we used the enhancer detector line rK781, which was isolated in a screen for *Antp*-regulated genes on the basis of their response to the overexpression of the ANTP homeoprotein in the eye-antennal disc (Wagner-Bernholz et al., 1991). We combined driver, responder and test constructs in larvae, exposed them to tetracycline treatment, and assayed β-galactosidase expression in wandering third instar larvae. When raised continuously with 10 μg/ml tetracycline, the normal pattern of rK781 expression was detected in all the discs (not shown) and in a few cells of the antennal disc (Fig. 6, left), as previously described (Wagner-Bernholz et al., 1991; Flister, 1991). When dissected from larvae that were removed from tetracycline exposure, *lacZ* expression could be reproducibly detected in the form of a crescent at the border between the arista and the third antennal segment (Fig. 6, middle). This area corresponds to the most proximal part of the wedge-shaped sector expressing *tTA*, as visualised with the *lacZ* reporter (Fig. 6, right) and is also the first to express *tTA* during third instar (Fig. 5B). These results show that derepression of *Antp* by removal of tetracycline can be demonstrated by the activation of a downstream target gene in the antennal disc. These findings indicate that the tetracycline-dependent expression system efficiently repressed *Antp* in embryos and allows subsequent derepression in imaginal discs.

DISCUSSION

In this study, we report a detailed evaluation of the different properties of the tetracycline-dependent gene expression system in *Drosophila*. Since its description in transformed HeLa cells (Gossen and Bujard, 1992) this regulatory system has been extensively used in cell culture. In higher eucaryotes including plants and mouse, tetracycline-controlled activity of *tTA* has been mostly evaluated on reporter genes (see Shockett and Schatz, 1996 for a review), although a few examples of successful expression of proteins have been reported (Efrat et al., 1995; Ewald et al., 1996; Mayford et al., 1996; Shockett et al., 1995; St-Onge et al., 1996). By using *Drosophila* lines expressing high levels of *tTA*, we show that the strong induction of the *lacZ* reporter can be efficiently prevented by tetracycline in both embryos and imaginal discs. We have evaluated the dose-response and defined easy and reliable protocols of tetracycline treatment to control the repression of the *lacZ* reporter gene. Furthermore, we also show that this system is fully functional to control the spatial and temporal expression of the ANTP homeoprotein. The lines *ey-tTA* and 205 described in this study show the highest levels of *tTA* among the lines generated to date in the laboratory and we have reproducibly obtained repression of gene activity in embryos by feeding their mothers with tetracycline in a range from 1 to 1000 μg/ml tetracycline, and in larvae, with as little as 0.1 μg/ml tetracycline. The use of tetracycline is especially appropriate to keep the inducible gene promoter silent during embryogenesis in order to direct its expression during larval development. Tetracycline concentrations ranging from 0.1 to 10 μg/ml ensure reactivation of the tetracycline-responsive promoter within 24 hours after transfer of the larvae to normal medium. It is important to point out that the amount of tetracycline required to inactivate *tTA* is both low and non-toxic. This is essential to keep the promoter inactive up to a desired stage and to ensure its fast activation upon removal from tetracycline exposure. We have found that the addition of tetracycline to the larval food does not give any toxic effect in a range of 0 to 100 μg/ml, although the development is slowed down at concentrations above 1 μg/ml. As shown with the *ey-tTA* strain, tetracycline can be used to control the timing of induction at distinct phases of development in order to define a phenocritical period. Temporal control of gene expression should also be effective during pupal development as a function of the concentration of tetracycline provided to the larvae before pupation. They can be well synchronised during this developmental period and go through a number of well-characterised stages (Ashburner, 1989). In combination with the use of the *lacZ* reporter, these features should help in determining the time course of induction of any gene driven by a *tTA*-expressing line of interest.

Our attempts to use the reverse tetracycline-controlled transactivator (*rtTA*, Gossen et al., 1995) have been unsuccessful in *Drosophila*. This transactivator is based on a mutagenized version of *tTA*, which binds the *tetO* sequences only in the presence of specific tetracycline derivatives. It corresponds to a 4-amino-acid exchange in *tetR*, which is thought to alter the conformation of the repressor and allows its binding to DNA upon association with certain tetracycline compounds (Gossen et al., 1995). Since it was originally isolated in a genetic screen in bacteria and tested successfully in mammalian cells (Gossen et al., 1995) and in transgenic mice (Kistner et al., 1996), *rtTA* might need a temperature close to 37°C to be stable. In contrast, *tTA* shows a potent activity in *Drosophila* and its negative regulation by tetracycline is not a major difficulty, as described above. Furthermore, both the repression and the kinetics of gene induction might be

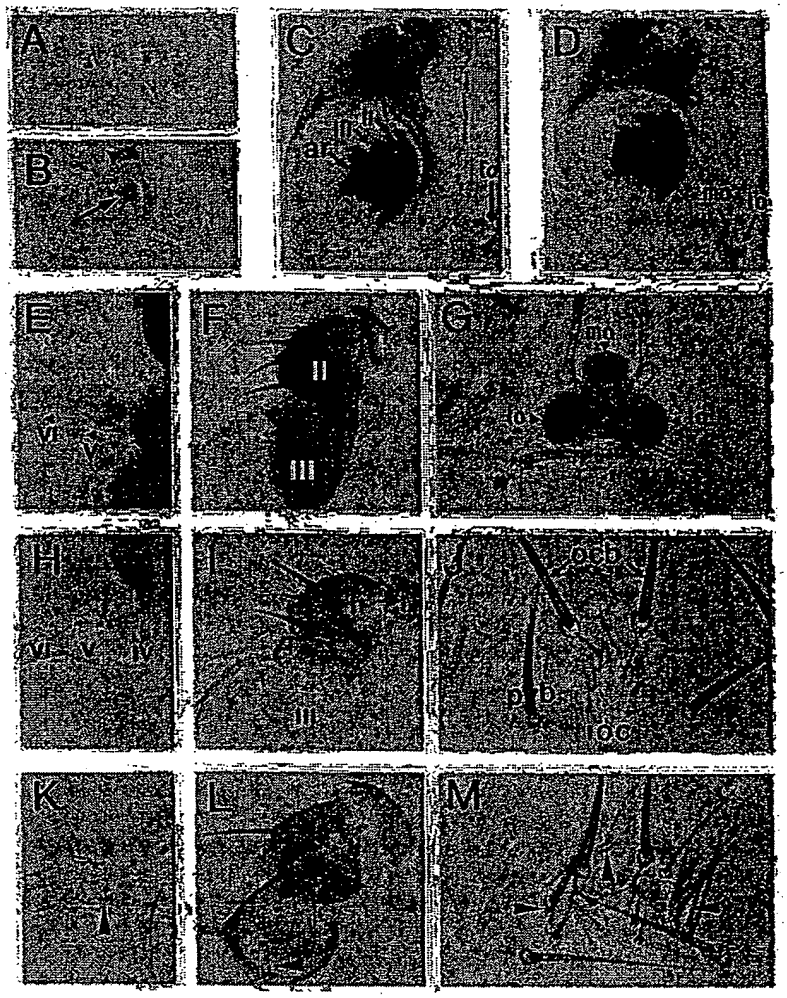

Fig. 5. Adult phenotype resulting from the directed expression of *Antennapedia* by the 205 strain. (A-D) Expression pattern of the 205 strain in the eye-antennal disc visualized by X-gal detection of β-galactosidase activity encoded by *tetO-lacZ* reporter. Discs are oriented with anterior up and dorsal left. (A) Early third instar. (B) Mid third instar; expression starts in the centralmost region of the antennal disc (arrow) and the presumptive palp region (arrowhead). (C) Late third instar larvae: expression has expanded in a wedge-shaped sector overlapping the most proximal region of the arista (ar) and the three major antennal segments (roman numerals). Expression in the lateral occellus (lo) that is not detectable in active wandering larvae is also indicated. (D) White prepupae: β-gal activity is detectable in the medium occellus (mo). (E-G) Expression of the *lacZ* reporter in the respective adult structures: the most proximal segments of the arista (E), the three antennal segments (F) and the occelli (G). (H-J) Normal phenotype of 205/+; *tetO-Antp*/+ adults raised continuously with 10 μg/ml tetracycline. Occipital, post vertical and interoccellar bristles are indicated by ocb, pvb and ioc, respectively. (K-M) Altered phenotype of 205/+, *tetO-Antp*/+ adults derived from larvae shifted from 10 μg/ml tetracycline to standard food during the late third instar. Note the thickening of the proximal segments of the arista (K, arrowhead), a bunch of new bristles on the third antennal segment and a modification in the number, the localization and the shape of the characteristic bristles of the second antennal segment (L, arrowheads). We could not determine the origin of these bristles on the basis of their morphology. Although different from the usual antennal bristles, they are not of a leg type because of the lack of the characteristic bract. The other main feature is a bunch of thick and long bristles of unknown origin close to the occelli (M, arrowheads).

increased further with one of the numerous tetracycline derivatives available. Some of them have been shown to be more potent effectors on *tTA* than tetracycline itself (Gossen and Bujard, 1993; Chrast-Balz and Hooft van Huijsduijnen, 1996).

Binary systems for controlling gene expression

The interest in using a binary system that combines an effector molecule for controlling activity of a responder promoter has been largely demonstrated with the GAL4/UAS system (Brand and Perrimon, 1993). Our method makes use of a similar experimental strategy, which allows the stable integration of any kind of construct in a parental fly strain in the absence of the transactivator. Tissue-specific activation of the gene construct is achieved in the offspring of a cross with a driver line chosen for its pattern of expression of the transactivator. The *tet* system provides a more versatile tool, owing to the possibility of controlling the timing of gene expression during development. In addition, the use of the vectors RHT and WTP facilitates the generation of driver strains expressing *tTA* under the control of previously isolated tissue-specific enhancers, and responder strains carrying any gene of interest under the regulation of the *tetO*-containing promoter. We also plan to generate a collection of *tTA*-expressing strains following the

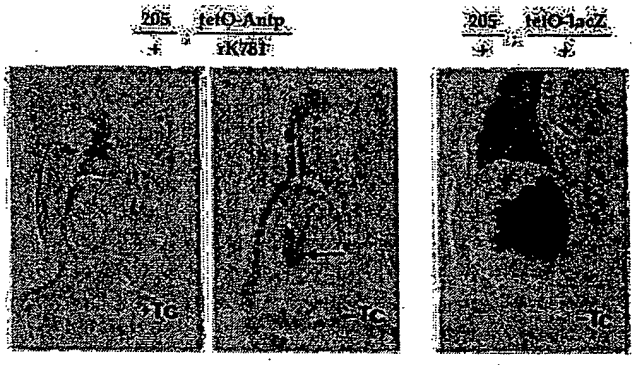

Fig. 6. Tetracycline-controlled expression of *Antennapedia* by the 205 strain activates rK781. Shown are β-galactosidase-stained antennal discs isolated from larvae raised continuously on 10 μg/ml tetracycline (+Tc) or shifted to standard food 48-72 hours before dissection (–Tc). The arrow points to the activation of rK781 in the centralmost region of the wedge-type sector of *tTA* expression, visualized on the right by the *tetO-lacZ* reporter. Eggs were collected over 12 hours from tetracycline-treated females of the genotype Cy[A405. M2]/205; rK781/rK781 mated with +/+ ; *tetO-Antp*/TM6,Tb males. Larvae of the genotype 205/+ ; *tetO-Antp*/rK781 were identified by their Tb^+ phenotype and the absence of the staining pattern due to the Cy[A405. M2] chromosome.

random integration into the genome of an enhancer detector construct with *tTA* as a reporter gene. The availability of strains expressing *tTA* in a wide variety of patterns will ensure a large number of applications for the *tet* system.

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ORIGINAL PAPER

Appendix B

E. T. Bieschke · J. C. Wheeler · J. Tower

Doxycycline-induced transgene expression during Drosophila development and aging

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Abstract The "reverse" tetracycline repressor (rtR) binds a specific DNA element, the tetracycline operator (tetO), only in the presence of tetracycline, or derivatives such as doxycycline (dox). Fusion of rtR to the transcriptional activation domain of herpes virus protein VP16 produces a eukaryotic transactivator protein (rtTA). rtTA has previously been shown to allow dox-dependent transcription of transgenes linked to tetO sequences in mammals. To adapt this system to *Drosophila*, the *Actin5C* promoter was used to drive constitutive expression of rtTA in transgenic flies. Three reporter constructs, each encoding *E. coli* β-galactosidase (β-gal), were also introduced into transgenic flies. In one reporter seven tetO sequences were fused to the *Adh* core promoter. The other two reporter constructs contain seven tetO sequences fused to the *hsp70* core promoter. Feeding of transgenic *Drosophila* containing the rtTA construct and any one of the three reporter constructs with dox caused up to 100-fold induction of β-gal. Dox induced β-gal expression in all tissues, in larvae and in young and senescent adults. Induction of β-gal in adults had no detectable effect on life span. These results suggest the potential usefulness of this system for testing specific genes for effects on *Drosophila* development and aging.

Key words Tetracycline repressor · Inducible promoter · Drosophila · Aging

Introduction

Inducible gene expression systems have long been an important tool in analyzing the function of specific genes in bacteria, yeasts, and *Drosophila*. In *Drosophila*, inducible transgenic systems usually rely wholly or in part on the use of a heat shock protein (hsp) gene promoter, which is transcriptionally induced in response to heat stress (Lis et al. 1983). While hsp gene promoters have been used to great advantage in many experiments, the system has several important limitations. First, the heat stress required for induction can have pleiotropic effects, including developmental abnormalities (phenocopies) (Lindquist 1986) and reduced fertility and viability. This is a problem particularly in experiments designed to study the aging process, since life span will be dramatically affected by changes in fertility and viability (Tower 1996). Another situation where the use of a heat-inducible promoter is problematic is the analysis of the heat shock proteins themselves. It is not possible to induce expression of a single hsp and study its effects, without the complication of inducing the entire endogenous repertoire of hsps. This problem has been overcome in certain experiments by using the metallothionein promoter (Petersen and Lindquist 1988; Solomon et al. 1991), which is inducible by heavy metal ions. However, the metallothionein promoter functions only in specific gut cells in transgenic *Drosophila* (Otto et al. 1987), thus limiting its potential usefulness. Therefore, there is a need for an alternative inducible gene expression system in *Drosophila*.

In the last five years, efficient inducible gene expression systems have been developed for mammalian systems based on the *E. coli* tetracycline repressor (tetR) (Gossen and Bujard 1992; Furth et al. 1994). The tetR binds to its target sequence, the tetracycline operator (tetO) only in the absence of the antibiotic tetracycline. The first system developed for mammals was the "tet-off" system (Gossen and Bujard 1992; Furth et al. 1994; Shockett et al. 1995). tetR protein was fused with the transcriptional activation domain of herpes virus transcription factor VP16. In the absence of tetracycline this protein binds to tetO sequences placed within the promoter of a gene of interest, thereby driving transcription. Addition of tetracycline then prevents binding and stops transcription ("tet-off"). A "tet-on" system was created

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by generating a mutant tetR:VP16 fusion protein, which had the reverse property of only binding to the tetO and activating transcription in the presence of tetracycline ("tet-on") (Gossen et al. 1995; Kistner et al. 1996). We report here the successful adaptation of the "tet-on" system to transgenic *Drosophila*.

Materials and methods

Plasmid constructions

Plasmid rtTA (reverse-tetracycline Trans Activator) was constructed by first inserting the 850-bp *Hin*dIII-*Xba*I fragment from pCaSpeR-AUG/β-gal (Thummel et al. 1988), containing the SV40 splice and poly(A) signals, into the *Hin*dIII (partial restriction digestion) and *Xba*I sites of the polylinker of the pCaSpeR4 transformation vector (Thummel and Pirotta 1992), to generate plasmid cSV. Plasmid pUHD172–1neo (Gossen et al. 1995) was digested with *Eco*RI, endfilled with T4 polymerase, then digested with *Bam*HI, to liberate a 1-kb fragment containing the reverse-tetracycline trans-activator coding sequence. Plasmid cSV was digested with *Spe*I, endfilled with T4 polymerase, then digested with *Bam*HI, and the 1-kb fragment from pUHD172–1neo was inserted, to generate the plasmid cTSV. DNA sequencing of cTSV revealed that it had resulted from an unexpected ligation event: the *Eco*RI site from the inserted fragment was conserved in this cloning step, and the 1-kb fragment was actually inserted into the *Bam*HI site, without any change in the *Spe*I site. The *Actin5C* promoter was inserted into plasmid cTSV in several steps. First, plasmid D237 (also called "Act5C > Draf+ > nuc-lacZ"; Struhl and Basler 1993) was digested with *Not*I, endfilled with T4 polymerase, then digested with *Kpn*I, and the resultant 4.3-kb fragment containing the *Actin5C* promoter was inserted into the *Kpn*I/*Eco*RV sites of pBlueScript II KS (Stratagene), to generate pAc. The 4.3-kb *Actin5C* promoter fragment was liberated from pAc by restriction digestion with *Kpn*I and *Eco*RI, and inserted into the *Kpn*I/*Eco*RI sites of cTSV, to generate the plasmid cATSV. DNA sequencing revealed that the *Actin5C* promoter in plasmid cATSV was in the wrong orientation relative to the reverse-tetracycline transactivator coding region. To correct this, the *Actin5C* promoter region was liberated by digestion with *Eco*RI, and then re-inserted into the same *Eco*RI site. DNA sequencing was used to identify a construct with the *Actin5C* promoter in the correct orientation, which was then named plasmid rtTA.

The seven tandem repeats of the tetO region in plasmid pUHC13–3 (Gossen et al. 1995) were amplified by PCR using the primers: 5′-TCGACTGCAGCTTTCGTCTTCAAGAATTCCTC-GAG-3′ and 5′-AGCTTCTAGATACACGCCTACTCGACCC-GGGTACCGAG-3′. The 367-bp PCR product was digested with *Pst*I and *Xba*I at the sites engineered into the primers, and then inserted into the *Pst*I/*Xba*I sites of pBlueScript II, to generate plasmid p7T.

Plasmid 7TAdh was constructed as follows. Plasmid pAdh/β-gal (Irvine et al. 1991; Koelle et al. 1991) was partially digested with *Eco*RI, and then completely digested with *Pst*I to liberate a 4.8-kb fragment containing the *Adh* basal promoter region (positions −33 to +53), the *Ubx* 5′ leader sequences fused to *lacZ*, and the SV40 splice and poly(A) signals. This fragment was cloned into the *Pst*I/*Eco*RI sites of the pCaSpeR4 polylinker, to generate plasmid pCaSpeR-Adh/β-gal. The 359-bp *Pst*I-*Xba*I fragment from plasmid p7T, containing the heptameric tetO region, was then inserted into the *Pst*I/*Xba*I sites of pCaSpeR-Adh/β-gal, to generate plasmid 7TAdh.

Plasmid 7T40 was constructed as follows. Construct c70Z (Simon and Lis 1987) was digested with *Hin*dIII and *Eco*RI to liberate a fragment containing the *hsp70* promoter fused to *E. coli lacZ*. This *Hin*dIII-*Eco*RI fragment was cloned into the *Hin*dIII/*Eco*RI sites of plasmid pBS2N to generate plasmid pBS2N′. Plasmid pBS2N is pBlueScript II KS + (Stratagene) in which the unique *Kpn*I site has been converted to a *Not*I site (a gift of L.R. Bell, University of Southern California). Construct c70Z was also digested with *Eco*RI alone to liberate an *Eco*RI fragment containing the *hsp70* poly(A) signal sequences, and this fragment was cloned into the unique *Eco*RI site of plasmid pBS2N′ to generate plasmid pBS2N″. Plasmid pBS2N″ was digested with *Hin*dIII and *Apa*I, treated with exonuclease III and with nuclease S1, and then ligated. The resultant plasmid was called c40Z, and DNA sequencing revealed a 5′ *hsp70* promoter deletion to position −40 relative to the start site of transcription. Plasmid c40Z is one of a series of *hsp70* 5′ promoter deletions which will be described in detail elsewhere (J. C. Wheeler and J. Tower, unpublished data). Plasmid c40Z was digested with *Not*I to liberate a 3.7-kb fragment containing the entire 5′Δ-40 *hsp70:lacZ* fusion gene, and this fragment was cloned into the *Not*I site of p7T, to generate plasmid p7T40-pre. A fragment containing the seven tetO repeats and the entire 5′Δ-40 *hsp70:lacZ* fusion gene was liberated from p7T40-pre by digestion with *Xho*I and *Spe*I, then inserted into the *Xho*I/*Spe*I sites in the polylinker of pCaSpeR4, to generate plasmid 7T40.

Plasmid 7TAUG was constructed as follows. A 4.6-kb SalI fragment from pCaSpeR-AUG/β-gal (Thummel et al. 1988), containing the *Adh* translation initiation sequence fused to *lacZ* and the SV40 splice and poly(A) signals, was cloned into the *Sal*I site of pBlueScript II KS, to generate plasmid pAUG. A *Pst*I fragment from plasmid 7T40, containing the seven tetO repeats and the *hsp70* promoter from −40 to +86, was inserted into the *Pst*I site of pAUG, to generate plasmid p7TAUG. A fragment containing the seven tetO repeats and the entire *hsp70:lacZ* fusion gene was liberated from p7TAUG by digestion with *Xho*I, and inserted into the *Xho*I site of pCaSpeR4, to generate plasmid 7TAUG.

Drosophila culture

Fly stocks were maintained on cornmeal/agar medium (Ashburner 1989). To obtain adult flies of defined ages, stocks were cultured at 25° C until 0–2 days post-eclosion, and then males only were transferred to 25° C or 29° C as indicated in Figure legends. These males were maintained at < 50 per vial and transferred to fresh vials every 2–4 days. Double transgenic adult males were obtained by crossing males of a transactivator stock (rtTA) to virgins of the reporter stocks (7TAdh, 7T40, and 7TAUG). Transgenic flies were generated by standard methods (Rubin and Spradling 1982), using the w^{1118} recipient strain.

Doxycycline treatments

Young flies (5–7 days post-eclosion) and old flies (28–32 days post-eclosion) were treated with the tetracycline derivative doxycycline hydrochloride (dox) (Sigma) by feeding. The indicated concentration of dox, in 20 mM Tris (pH 7.5) containing 10% sucrose, was soaked into a single Kim-Wipe (Kimberly-Clark), in an empty *Drosophila* culture vial. After feeding with dox for the specified time, the flies were returned to cornmeal/agar food vials, and allowed to recover as indicated. For treatment of larvae, the cornmeal/agar medium was supplemented with dox to a final concentration of 0.25 mg/ml, prior to seeding of the culture.

Spectrophotometric assay of β-galactosidase activity

β-Galactosidase (β-gal) activity was quantitated in whole fly extracts using published procedures (Simon and Lis 1987). Assays were performed under conditions in which the reaction was linear with regard to the amount of extract. Data are presented as the average +/− the standard deviation for triplicate assays. Protein concentration of extracts was determined using the Bradford reagent (BioRad). The w^{1118} strain was used to generate all transgenic lines, and no β-gal activity was detectable in extracts of the w^{1118} strain using the spectrophotometric assay.

In situ staining for β-galactosidase activity

β-galactosidase expression was visualized in dissected flies, larvae, and cryostat sections using published procedures (Simon et al. 1985).

Results

Basic components of the system

To achieve tetracycline-inducible induction of transgenes in all tissues it is necessary that the reverse tetracycline transactivator (rtTA) be expressed in all tissues. The rtTA is a fusion of the reverse tetracycline receptor (rtR), which binds to DNA only in the presence of tetracycline, with the transcriptional activation domain of herpes virus transcription factor VP16. In construct rtTA, the constitutive *Drosophila Actin5C* promoter was used to drive expression of the rtTA coding region. This construct also contains the SV40 poly(A) signal sequence (Fig. 1A). To test the system, three reporter constructs were generated, each encoding *E. coli* β-gal. The constructs differed in the source of the core promoter, 5′ UTR, and polyadenylation signal sequences in order to maximize the chances of generating a construct which could yield high-level transgenic protein expression in *Drosophila*. In the first reporter construct (7TAdh) seven tetO sequences are fused to the *Adh* core promoter, followed by the *Ubx* 5′ untranslated region, the *E. coli lacZ* coding region, and the SV40 poly(A) signal (Fig. 1B). A regulatory element composed of seven tetO sequences was chosen because this element was previously shown to function in transgenic mice (Kistner et al. 1996). In the second reporter (7T40), the seven tetO sequences are fused to the *hsp70* core promoter, and *hsp70* 5′ untranslated region, followed by the *E. coli lacZ* coding sequences and the *hsp70* poly(A) signal (Fig. 1C). In the third construct (7TAUG), the seven tetO sequences are fused to the *hsp70* core promoter, followed by the *Adh* 5′ untranslated region, the *lacZ* coding region, and the SV40 poly(A) signal (Fig. 1D). Multiple independent transgenic lines were generated for each construct. Each line is homozygous for the transgenic construct, and is designated by the name of the construct followed by the chromosome in which the construct is inserted (in parenthesis), followed by a letter/number combination for each independent transgenic line. For example, line 7TAdh(2)A2 is transgenic line number A2 and has the 7TAdh construct inserted on the second chromosome.

Flies were then generated which contained both the rtTA construct and the 7TAdh construct ("double-transgenic" flies). This was done by crossing flies of stock rtTA(2)C1 to flies of stock 7TAdh(2)A2, which yields progeny containing one copy of each construct. A sample of these double transgenic flies were fed sucrose solution containing 1.0 mg/ml dox for 48 h, while the controls were fed sucrose solution alone. The flies were allowed to recover for 3 days, then sectioned using a cryostat, and the sections were stained for β-gal activity (Fig. 2A). In the treated flies robust β-gal activity (blue stain) was detected in all tissues. In the control flies, low-level β-gal activity was detected primarily in the gut, and thus the system allows dox-induced transgene expression in all tissues of the adult. The same results were obtained with transgenic flies containing the other two reporter constructs, 7T40 and 7TAUG (data not shown).

To determine if the system also works during development, line rtTA(2)C1 was crossed again to reporter line 7TAdh(2)A2, and also to reporter line 7T40(3)B1, and the larvae from each cross were cultured on food containing 0.25 mg/ml dox, and on

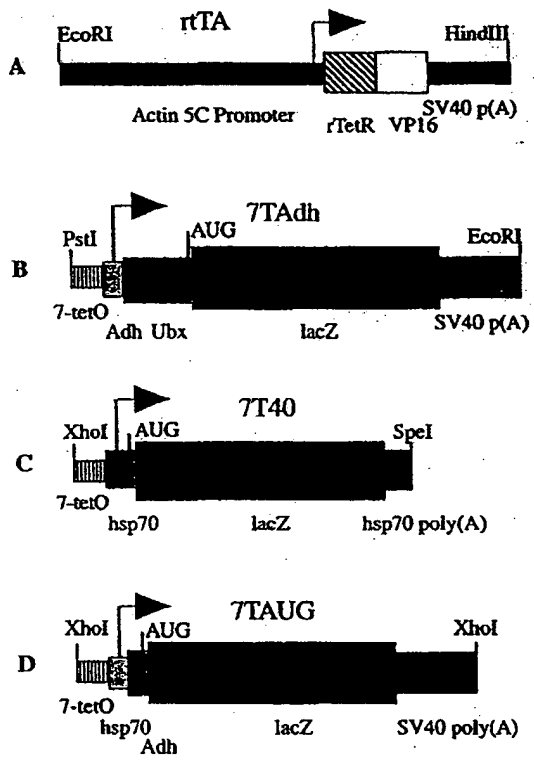

Fig. 1A–D Transgenic constructs. Each construct fragment shown is cloned into the indicated restriction sites of the polylinker of the pCaSpeR-4 transformation vector. The assembly of each construct is described in detail in Materials and methods. Diagrams are not to scale. **A** rtTA. The constitutive *Actin5C* promoter and 5′ untranslated region are fused to the coding sequences for the rtTA (reverse tetracycline transactivator), which is a fusion of the rtR (reverse tetracycline repressor) and the transcriptional activation domain of herpes virus protein VP16. The poly(A) signal sequences are from SV40. **B** 7TAdh. Reporter construct consisting of seven tetO sequences, the *Adh* core promoter, the *Ubx* 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region and the SV40 poly(A) signal sequences. **C** 7T40. Reporter construct consisting of seven tetO sequences, the *hsp70* core promoter, 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region and the *hsp70* poly(A) signal sequence. **D** 7TAUG. Reporter construct consisting of seven tetO sequences, the *hsp70* core promoter, the *Adh* 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region, and the SV40 poly(A) signal sequence

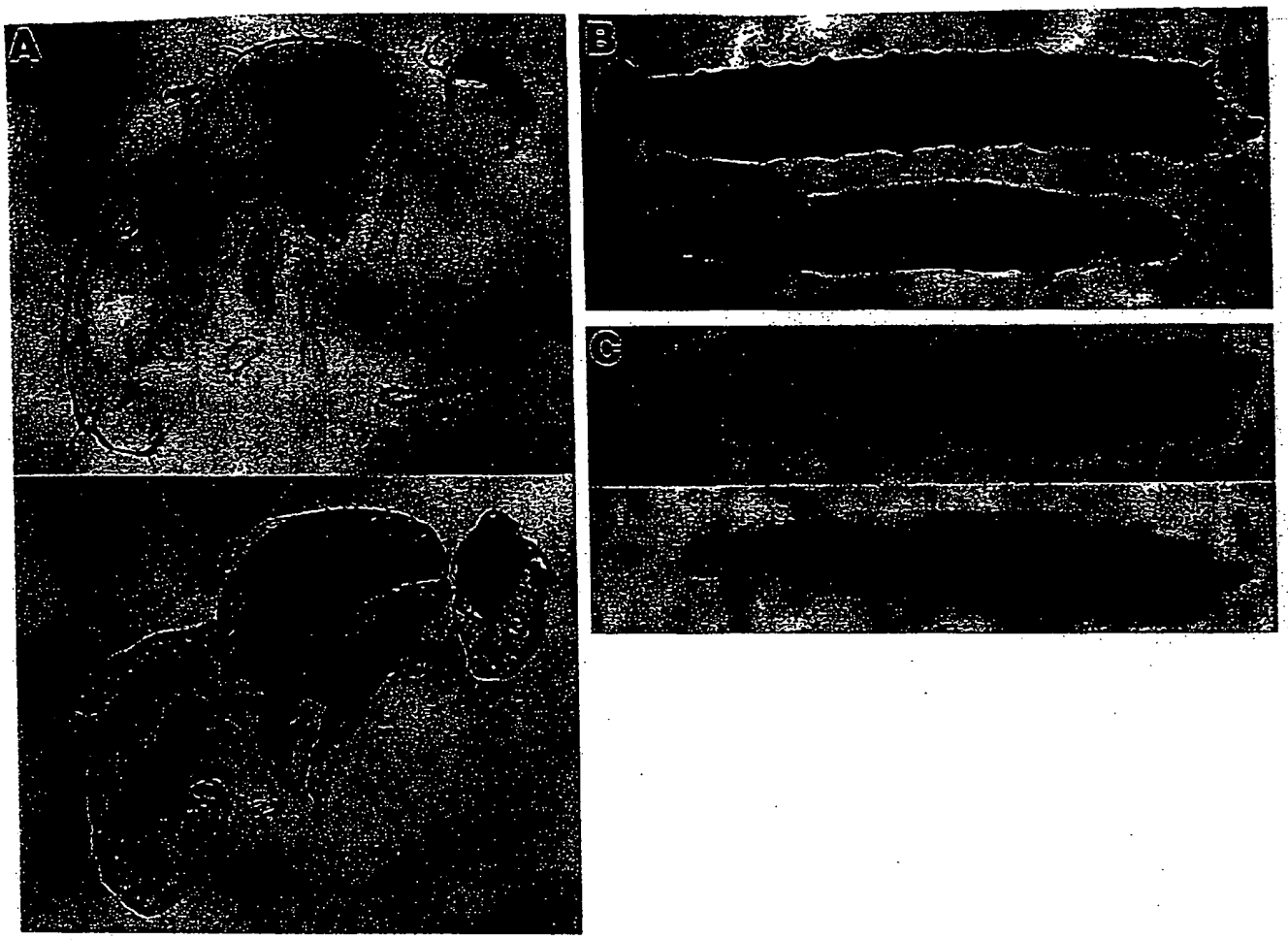

Fig. 2A–C Dox-induced transgene expression detected by an in situ β-gal activity assay. A rtTA transgenic line rtTA(2)C1 was crossed to reporter line 7TAdh(2)A2. Young adult progeny were fed with either control sucrose solution (*upper panel*) or sucrose solution containing 1.0 mg/ml dox (*lower panel*) for 48 h, and then allowed to recover for 3 days. Flies were sectioned on a cryostat, and stained for β-gal activity using the chromogenic substrate X-gal. In the control (*upper panel*), low-level β-gal activity is detected primarily in gut tissues. The gut staining indicates some leakiness of expression in the absence of dox, as in non-transgenic *Drosophila* only very faint gut staining is detectable, and only in the abdomen (data not shown, see also Wheeler et al. 1995). In dox-treated (*lower panel*), β-gal activity is detected in all tissues, with the exception of the central region of the indirect flight muscles. All of the indirect flight muscle tissue stains intensely if the staining reaction is allowed to continue for a longer period (data not shown). However with longer staining times the increased intensity of stain in the other body segments obscures the detail of specific tissues, and therefore the results for the shorter staining time are presented. B Progeny from the cross rtTA(2)C1 × 7T40(3)B1 were cultured on standard *Drosophila* culture media (upper larva) or *Drosophila* media containing 0.25 mg/ml dox (lower larva). Whole third-instar larvae were stained in situ for β-gal activity. No β-gal activity was detected in the control tissue larvae (*upper larvae*), or in non-transgenic larvae (data not shown). General β-gal activity was detected in the dox treated larvae (*lower larvae*). C Repeat of the experiment in B, using progeny of the cross rtTA(2)C1 × 7TAdh(2)A2. β-Gal expression in larvae with this reporter was reproducibly less efficient than in the experiment shown in B

control food. As seen in Fig. 2B, C, staining of whole third-instar larvae revealed high-level, tissue general induction of β-gal activity with reporter 7T40(3)B1, and somewhat lower level, tissue general induction with reporter 7TAdh(2)A2. The dox-fed larvae were also observed to be slightly smaller than the controls, which may be due to a toxic effect of the dox and/or β-gal expression during development.

Characterization of the response

The induction of β-gal expression can be quantitated by spectrophotometric assay of β-gal activity in fly extracts. This assay was used to optimize the time course of dox treatment. Transactivator line rtTA(2)C1 was crossed to reporter line 7TAdh(3)D1, and the double transgenic progeny were treated with 1.0 mg/ml of dox for 24 h, 48 h, and 48 h plus varying times of recovery without dox. As seen in Fig. 3A, 48 h of treatment plus 3 days of recovery gave the optimal degree of induction (~10-fold). With greater times of recovery, β-gal activity decreased, indicating that the induction is reversible upon withdrawal of dox. The same result was obtained using a different reporter stock, containing the 7T40

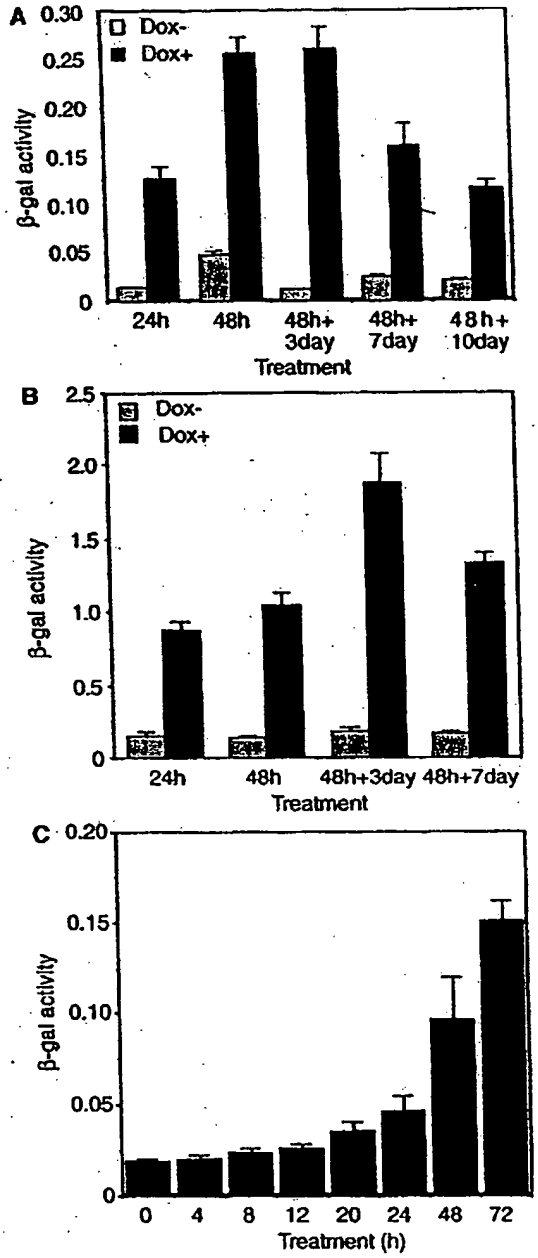

Fig. 3A–C Time course of transgene induction by dox. A Young adult progeny of the cross rtTA(2)C1 × 7TAdh(3)D1 were mock treated (Dox-; *stippled bars*), or treated with 1.0 mg/ml dox (Dox+; *black bars*) for the indicated time periods, and allowed to recover as indicated. Triplicate samples containing three flies each were homogenized, and β-gal activity was quantitated using the spectrophotometric assay. β-Gal activity is expressed in relative units, and the averages +/- SD are presented. B The experiment in A was repeated with a different reporter stock, using young adult progeny from cross rtTA(2)C1 × 7T40(2)E1. C The experiment in A was repeated using progeny from cross rtTA(2)C1 × 7TAdh(2)A2, and the timecourse for induction was analyzed in greater detail

construct, 7T40(2)E1 (Fig. 3B). Thus, both the *hsp70* core promoter and the *Adh* core promoter can respond to activation by the tetO sequences and the rtTA transactivator.

In transgenic mice the activation by the rtTA transactivator can be quite rapid, with activation by several orders of magnitude occurring in the first 4 h, and maximum levels of activation being achieved by 24 h (Kistner et al. 1996). The timecourse of activation in *Drosophila* was analyzed in greater detail (Fig. 3 C), and found to be significantly slower. In the progeny of the cross rtTA(2)C1 × 7TAdh(2)A2, induction of β-gal by dox feeding was quantitated at intervals between 4 and 72 h. Significant activation was not detected until 8–20 h, and maximal induction required ≥72 h. Similar results were obtained with construct 7TAUG (data not shown). Note that while the level of induction and timecourse was similar for the different reporters in Fig. 3, they are not identical. This probably reflects small differences in the activities of the different reporter insertions, as well as the variability inherent in working with live adult *Drosophila* and administration of dox by feeding.

The *Drosophila* tet-on system was next characterized for the dose response to dox (Fig. 4). Double transgenic adults (rtTA(2)C1 × 7TAdh(2)A2) were fed dox for 48 h and allowed to recover for 3 days (Fig. 4, open circles), or for 96 h plus a 3-day recovery period (closed circles). For 48-h treatment times, β-gal activity was found to increase in response to dox concentrations from 0.01 to 2 mg/ml. Use of the longer 96-h treatment time allowed equivalent levels of β-gal expression with one-tenth as much dox. Thus, longer treatment times reduce the amount of dox required for efficient induction.

To compare the relative activities of the three different reporter constructs, two independent transgenic lines for each reporter were crossed to the rtTA(2)C1 transactivator line (Fig. 5A). Dox-induced β-gal expression

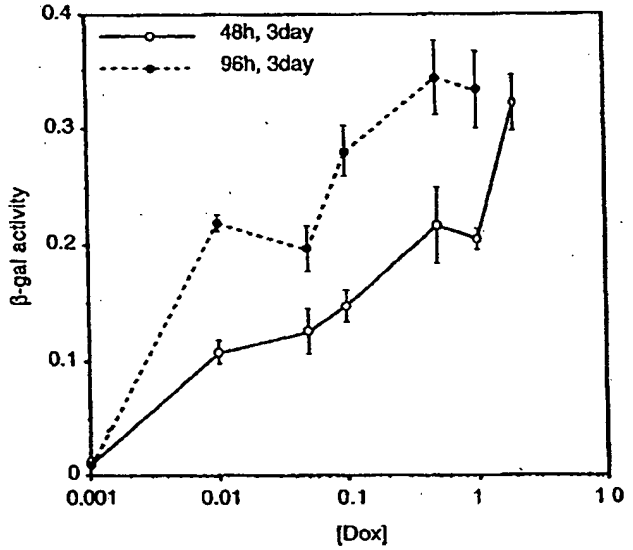

Fig. 4 Dose response of transgene induction by dox. Young adult progeny of cross rtTA(2)C1 × 7TAdh(2)A2 were fed the indicated concentrations of dox for 48 h and allowed to recover for 3 days (*open circles*), or for 96 h plus a 3-day recovery period (*closed circles*). β-Gal expression was quantitated as in Fig. 3

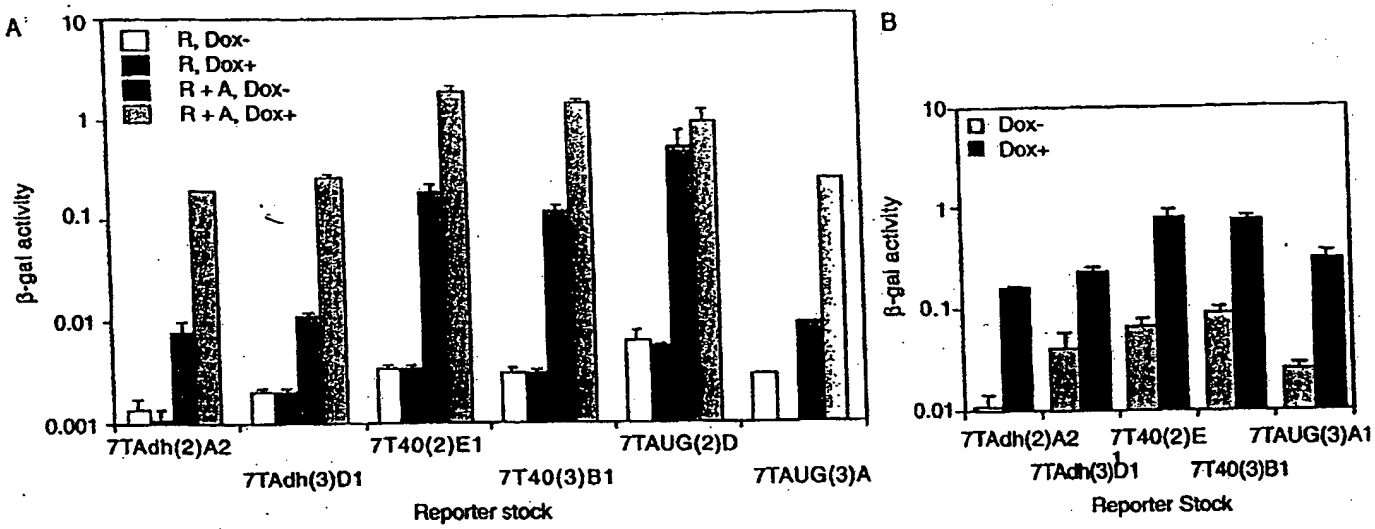

Fig. 5A, B Comparison of transgenic reporter constructs and lines. All dox treatments were for 48 h plus 3 days recovery. A Assay in young adults. The indicated reporter lines (R, reporter alone) were assayed with and without dox treatment, as indicated. Each indicated reporter line was also crossed to the rtTA line rtTA(2)C1, and the progeny (R+A, reporter plus activator) were assayed with and without dox treatment, as indicated. B Assay in old adults. rtTA line rtTA(2)C1 was crossed to each indicated reporter line, and old adult progeny were assayed with and without dox treatment, as indicated. β-Gal expression was quantitated as in Fig. 3

was observed with all three constructs, with induction factors ranging from 12- to 25- fold. In general, the 7T40 reporter construct gave higher levels of β-gal expression than the other two reporter constructs; however, the background expression in the absence of dox was also higher. Thus, the induction factor achieved was similar for each of the three reporter constructs.

To determine if the system functions during aging of *Drosophila*, the activity of each reporter construct was also assayed in senescent (30-day-old) flies (Fig 5B). Each reporter was found to support dox-induced β-gal expression in senescent flies, with induction factors ranging from 8- to 15-fold.

The dox-inducible system is dependent upon efficient, general expression of the transactivator construct, rtTA. Because the chromosomal site of insertion of the rtTA transgene can affect the level of expression, different independent rtTA transgenic lines may vary in their activity. To compare their activities, each of 13 independent rtTA transgenic lines was crossed to the 7TAdh(3)D1 reporter, and the efficiency of induction of β-gal by dox was quantitated (Fig. 6A). The different independent rtTA lines were found to vary in activity, both with regard to the amount of background β-gal activity in the absence of dox, and with regard to the maximum level of induction in the presence of dox. Transgenic transactivator line rtTA(3)E2 appeared to be the best: in the absence of dox, background β-gal levels were as low as in flies carrying the reporter construct in the absence of any transactivator, and dox treatment yielded a 40-fold induction. To confirm this result, each

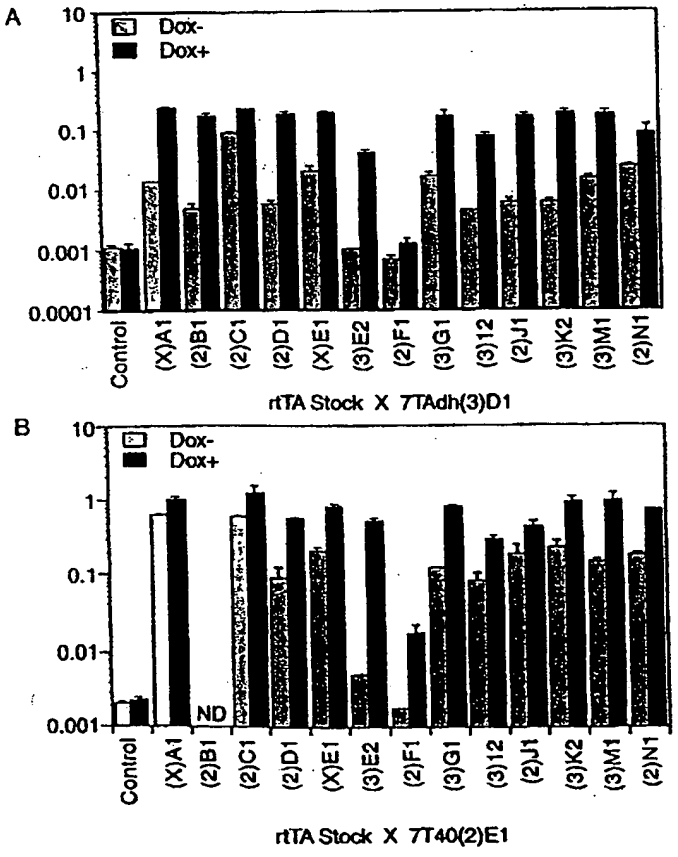

Fig. 6A, B Comparison of different transgenic transactivator (rtTA) lines. All dox treatments were for 48 h plus 3 days recovery. A The indicated rtTA lines were each crossed to reporter line 7TAdh(3)D1. The young adult progeny from each cross were assayed without dox treatment (Dox-; *stippled bars*), and with 1.0 mg/ml dox treatment (Dox+; *black bars*), as indicated. Control was the reporter line 7TAdh(3)D1 alone. B The experiment in A was repeated using the reporter line 7T40(2)E1. ND, not done

transactivator stock was also tested in combination with reporter stock 7T40(2)E1 (Fig. 6B). Again the various transactivator lines varied with regard to background and maximal level of induction, and their activity relative to each other was similar to that observed using the 7TAdh reporter. Line rtTA(3)E2 was again found to have the lowest background, and to be the most active, yielding 100-fold induction of β-gal in response to dox.

One potential use of the dox-inducible system in *Drosophila* is in the analysis of the effects of specific genes on the aging process. Ideally, for such experiments, the system itself should not have any effect on life span. To characterize the system for effects on life span, several genetic backgrounds were tested for longevity, with and without dox feeding. Wild-type flies exhibited no negative effects on life span when fed with 0.1 mg/ml dox (Fig. 7A). Double transgenic flies (rtTA(2)C1 × 7TAdh(2)A2) expressed high levels of β-gal in response to 0.1 mg/ml dox (Fig. 4), and this expression also had no detectable negative effects on lifespan (Fig. 7A). Finally, a different combination of transactivator and reporter were tested. Transactivator rtTA(X)A1 was crossed to reporter 7TAdh(3)D1 and age-synchronized cohorts of adult flies were treated throughout their adult lifespan with no dox, 0.01 mg/ml dox, or 1.0 mg/ml dox (Fig. 7B). Treatment with 1.0 mg/ml dox was found to have a negative affect on life span. However, treatment with 0.01 mg/ml dox had no detectable affect on life span. The same results were obtained with several other combinations of rtTA and reporter lines (data not shown). Since 0.01 mg/ml dox and 0.1 mg/ml dox allow high-level induction of β-gal (Fig. 4), and have no detectable effect on lifespan (Fig. 7), these results suggest that the system should be useful for assaying the effects on life span of overexpression of specific genes.

Discussion

The hybrid transcriptional activator (rtTA) consisting of the rtR fused to the transcriptional activation domain of the herpes virus protein VP16 has previously been shown to be capable of supporting dox-induced transcription in transgenic mice. The experiments presented

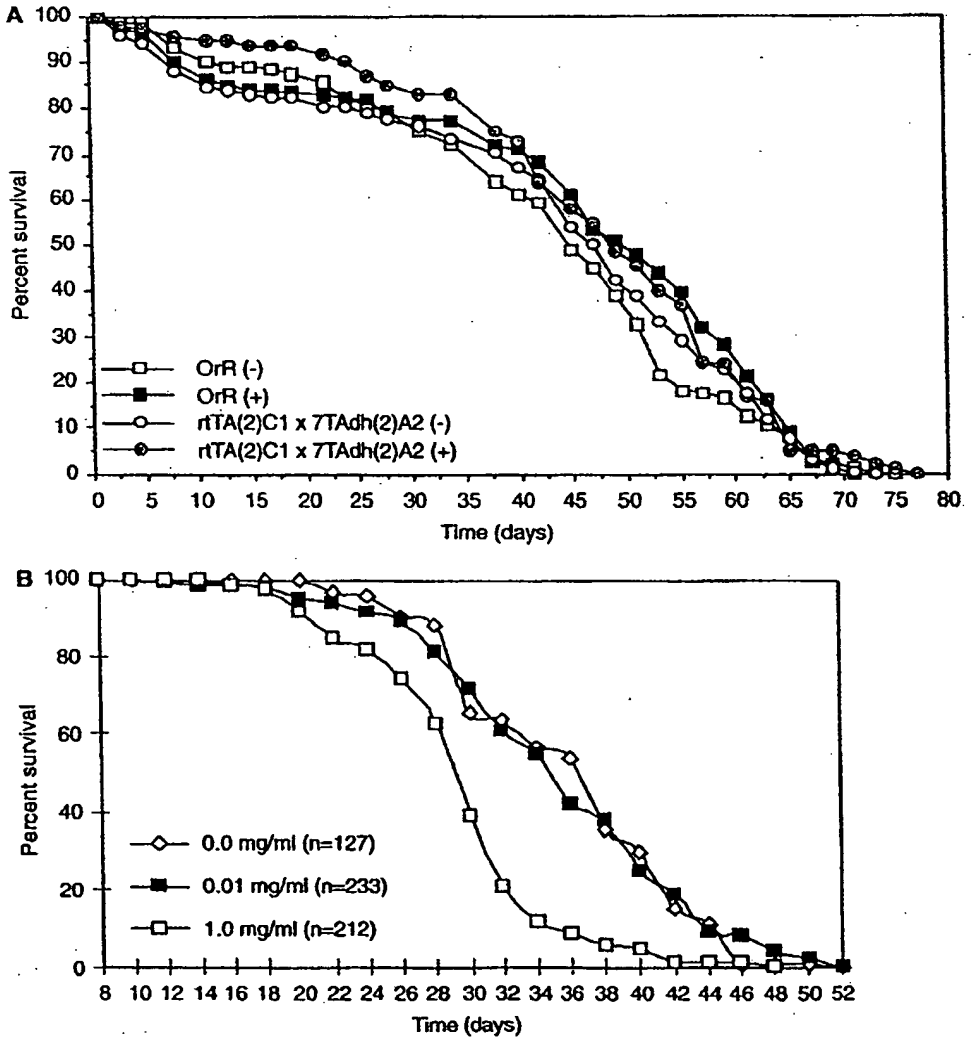

Fig. 7A, B Affect of dox-induced β-gal expression on *Drosophila* adult life span. A Wild-type Oregon R strain flies were treated throughout their adult lifespan either without dox (*open squares*) or with 0.1 mg/ml dox (*filled squares*), at 25° C. Flies were fed or mock-fed dox for 2 days, and then allowed to recover on standard media for 2 days, and this regimen was repeated until all the flies had died. The percentage of flies surviving is plotted as a function of time in days. The same experiment was performed using progeny from cross rtTA(2)C1 × 7TAdh(2)A2 grown in the absence of dox (*open circles*) or in the presence of 0.1 mg/ml dox (*closed circles*). At least 200 flies were used for each of the four survival curves. B Progeny from the cross rtTA(X)A1 × 7 TAdh(3)D1 were grown throughout their adult life span in the absence of dox (*open diamonds*), or in the presence of 0.01 mg/ml dox (*filled squares*), or 1.0 mg/ml dox (*open squares*), at 25° C. The number of flies used (n) for each survival curve is indicated. The different genotypes assayed in A and B vary in life span relative to each other, which is not unexpected due to the large effects of genetic background on life span (Curtsinger et al. 1995; Tower 1996)

here demonstrate that this rtTA functions in transgenic *Drosophila*, and can activate transcription at both the *hsp70* and *Adh* core promoters when they are linked to tetO sequences. Since all three reporter constructs performed similarly, the results suggest that in this system there is no significant difference in the effectiveness of the SV40 poly(A) signal relative to the *hsp70* poly(A) signal, and no significant difference between the effectivness of the *Adh*, *hsp70* and *Ubx* 5′ UTR regions. Dox-induced transgene expression was detected in all tissues, and induction ranged from 10- to 100-fold. Different transgenic lines containing the rtTA construct varied considerably in activity. Variation was observed in the maximal level of induction achieved, and in the amount of background activity observed in the absence of dox. This variation is likely to be due to chromosomal position effects on the expression of the rtTA transposon, and perhaps to other differences in the genetic background of the lines. The different transgenic lines of the reporter constructs also varied in activity, most probably for the same reasons. The maximum degree of induction that was achieved was 100-fold. This is dramatically less than the five orders of magnitude induction obtained with the tet-on system in transgenic mice (Kistner et al. 1996). The maximal induction achieved in *Drosophila* is limited by at least two factors: First, the reporter constructs are slightly leaky, in that variable, low-level β-gal activity is detected even in the absence of the rtTA transactivator. Second, the rtTA transactivator appears to be partially active even the absence of dox treatment, in that reporter plus transactivator was often more active than reporter alone. The first problem might be addressed by protecting the reporter constructs from position effects with insulator elements (Roseman et al. 1993), and/or by identifying a less leaky core promoter. The second problem can be mitigated by identifying particular rtTA lines, such as rtTA(3)E2, which exhibit less background activation. Finally, we hypothesize that the herpes virus VP16 transcriptional activation domain used to create the rtTA transactivator may be better suited to interaction with the mammalian transcriptional machinery than with the *Drosophila* transcriptional machinery. This possibility may also be relevant to the slower time course of induction observed in *Drosophila*.

Despite its limitations, this inducible system has several potential advantages relative to the use of heat shock gene promoters. The dox-inducible system should be useful for studying hsps, as it will allow the investigator to induce the expression of a single hsp, and potentially inhibit its expression with antisense RNA, without inducing the endogenous heat shock response. The dox- inducible system also allows the investigator to induce a gene of interest at any time during the life cycle. This is particularly relevant to study of the aging process (Curtsinger et al. 1995; Tower 1996), where it is often desirable to alter gene expression specifically in the adult. For example, constitutive over-expression of Cu/Zn SOD may have beneficial effects on *Drosophila* life span but it also appears to have toxic affects during pupal development (Reveillaud et al. 1991). Using the dox-inducible system it should be possible to avoid toxic effects during development and cause over-expression of transgenes specifically in the adult where beneficial effects on life span may be more apparent.

The dox-inducible system should be readily adaptable to tissue-specific induction. Replacement of the constitutive *Actin5C* promoter in the transactivator construct rtTA with a tissue-specific promoter should provide tissue-specific expression of the rtTA transactivator and thus tissue-specific induction of the reporter. An elegant system for tissue-specific expression of the yeast GAL4 transactivator has been developed for *Drosophila* (Brand and Perrimon 1993; Brand and Dormand 1995). In this case tissue-specific expression of the GAL4 transactivator is driven by an "enhancer-trap" system: the transactivator is under the control of a weak transcriptional promoter which can become activated in a tissue- and temporal-specific manner when the P element inserts near transcriptional enhancer sequences in the chromosome. The large variety of tissue- and temporal-specific GAL4 transactivator expression patterns generated thus allows tissue- and temporal-specific expression of "reporter" type constructs containing GAL4 binding sites in their promoters. This system could be adapted to drive expression of the rtTA transactivator, thus creating a large variety of tissue-specific expression patterns inducible by dox.

Finally, it may be possible to create dox-dependent mutations in *Drosophila*. P element constructs with transcriptional promoters directed out of the end of the P element can cause over-expression and/or mis-expression of genes near the site of insertion, sometimes causing dominant mutations (Rorth 1996; Hay et al. 1997). Creation of a P element with a dox-inducible promoter directed out of the P element into flanking DNA sequences should sometimes cause dox-induced over-expression of a gene near the insertion site. This method should thus yield conditional (dox-dependent), dominant, gain-of-function mutations which would be useful for many types of genetic analyses; such experiments are now underway.

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Functional Characterization of the Enhancer Blocking Element of the Sea Urchin Early Histone Gene Cluster Reveals Insulator Properties and Three Essential *cis*-acting Sequences

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Insulator elements can be functionally identified by their ability to shield promoters from regulators in a position-dependent manner or their ability to protect adjacent transgenes from position effects. We have previously reported the identification of a 265 bp *sns* DNA fragment at the 3′ end of the sea urchin H2A early histone gene that blocked expression of a reporter gene in transgenic embryos when placed between the enhancer and the promoter. Here we show that *sns* interferes with enhancer-promoter interaction in a directional manner. When *sns* is placed between the H2A modulator and the inducible *tet* operator, the modulator is barred from interaction with the basal promoter. However, the *tet* activator (tTA) can still activate the promoter, even in the presence of *sns*, demonstrating that *sns* does not interfere with activity of a downstream enhancer. In addition, the H2A modulator can still drive expression of a divergently oriented transcription unit, suggesting that *sns* does not inhibit binding of transcription factor(s) to the enhancer. To identify *cis*-acting sequence elements within *sns* which are responsible for insulator activity, we have performed *in vitro* DNase I footprinting and EMSA analysis, and *in vivo* functional assays by microinjection into sea urchin embryos. We have identified three binding sites for protein complexes: a palindrome, a direct repeat, and a C + T sequence that corresponds to seven GAGA motifs on the transcribed strand. Insulator function requires all three *cis*-acting elements. Based on these results, we conclude that *sns* displays properties similar to the best characterized insulators and suggest that directional blocking of enhancer-activated transcription by *sns* depends on the assembly of distinct DNA-protein complexes.

Keywords: histone genes; enhancer blocking; insulator; H2A enhancer; microinjection

Introduction

Proper temporal and spatial regulation of gene expression requires the orderly and efficient interaction of transcription factors with their cognate sites. The elucidation of how this might occur is one of the major challenges in molecular biology. If transcription units are organized into independent functional domains, enhancers could activate transcription from a promoter within the same domain but would be restrained from interacting with promoters in external domains. Insulators seem to be involved in the organization of the eukaryotic genome into domains of gene expression.[1–4] Insulators have been identified because they interfere with

†The first two authors contributed equally to this work.

Abbreviation used: EMSA, electrophoretic mobility shift assay.

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enhancer-promoter communication only when positioned between them; for this reason they are generally called enhancer blocking elements. Although mechanisms by which these elements function in the normal context are not fully understood, it is thought that binding of chromosomal proteins to specific insulator sequences and subsequent protein-protein interactions can directionally restrict enhancer activity (for recent reviews see:[5,6]). The best characterized of these enhancer blocking elements are the *Drosphila*, scs, scs' and gypsy elements,[7-10] and the chicken HS4 insulator.[11,12] In addition to enhancer blocking activity, insulators can protect a transgene from chromosomal position effects when placed in flanking positions. However, only the chicken HS4[13,14] and the *Drosphila*, gypsy[15] elements seem to confer a barrier which prevents heterochromatin from spreading. Gypsy elements can also buffer a promoter from the silencing effect of the polycomb responsive element (PRE).[16] Elements which counteract silencing and act as heterochromatin boundaries have also been identified in yeast.[17-19] However, it is not known whether the yeast elements can also act as insulators of enhancer activity or display only chromatin boundary functions.[20]

Insulator DNA elements seem to be present in the tandemly repeated sea urchin early histone genes. The five early histone genes are expressed coordinately after meiotic maturation of the egg, and in early cleavage embryos until the blastula stage.[21] Although there is a single enhancer within the histone repeat unit, the 30 bp modulator of the H2A gene[22,23] each gene within the repeat is apparently regulated by gene-specific transcriptional elements..[24-26] From this observation, we hypothesized that there might be chromosomal elements which would direct and restrict H2A modulator function to its cognate H2A promoter. Subsequently, we identified a 265 bp sequence at the 3' end of the H2A early histone gene (see map in Figure 1), that, in microinjected sea urchin embryos, showed a blocking activity when placed between the enhancer and the promoter. We termed this sequence silencing nucleoprotein structure (*sns*). Although proof of the directionality of enhancer blocking activity was lacking, experimental evidence suggested that *sns* is an insulator of enhancer function rather than a general repressor of transcription. *sns* blocked enhancer-promoter interaction only when interposed, in either orientation, between a multiple array of the H2A modulator/enhancer and the basal thymidine kinase (tk) promoter. No other position influenced transgene expression. Furthermore, *sns* interfered with enhancer function but not with the activity of the basal promoter, in that it mantained the capacity to silence transgene expression when it was placed at a distance of 2.7 kb from the promoter.[28] Interestingly, our results suggested evolutionary conservation of enhancer blocking mechanisms, as *sns* was able to shield promoters from viral enhancers in transient and stably transfected human cell lines, as well as in *Xenopus laevis* oocytes[28] (unpublished observations).

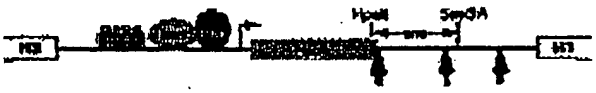

Figure 1. Map of the H2A transcription unit. CCAAT (CBF), modulator (MBF), putative GAGA factor binding sites in the H2A promoter, and the transcription start site are indicated. The *sns* fragment spanning from the *HpaII* to the *Sau3AI* restriction sites starts 14 nucleotides upstream of the stop codon of the H2A gene and resides in a region where three micrococcal nuclease hypersensitive sites (vertical thick arrows) appear at gastrula stages.[27]

Here we have further characterized the properties of the sea urchin *sns* DNA fragment. We present evidence demonstrating that *sns*, as in the case of the best characterized insulators, affects only enhancers located distally from the promoter and displays a directionality in enhancer blocking activity. In addition, we report the identification of three protein binding sites within *sns* that, as demonstrated by functional assays in transgenic sea urchin embryos, are collectively required for insulator activity.

Results

sns interferes with enhancer function in a directional manner

Utilizing RNase protection assays, we previously demonstrated the ability of *sns* to inhibit enhancer-activated expression of a transgene in microinjected sea urchin embryos only when interposed between an array of the H2A modulator/enhancer and the *tk* promoter.[28] Because basal expression from *tk* or other viral promoters in sea urchin is negligible, effects on transcription were dramatic, with previously abundant transcript levels becoming undetectable. We have used the same approach to investigate whether *sns* evinces other behaviors expected of insulators. We placed *sns* between two enhancers in a chloramphenicol-acetyl transferase (CAT) gene transcription unit (Figure 2(a), construct 2). As a distal enhancer, we used the sea urchin H2A modulator array, containing at least four binding sites for the MBF-1 *trans*-activator; the proximal enhancer was the heptamerized tetracycline (*tet*) operator. In HeLa cells, the *tet* operator is induced upon binding of the Tet repressor-VP16 activation domain chimera (tTA *trans*-activator) and stimulates transcription of a reporter gene by several orders of magnitude.[29] The *tTA* gene was placed under the control of the multiple modulator elements and the *tk* minimal promoter (construct A). This construct was coinjected with the *CAT* reporter gene driven by the *tet*

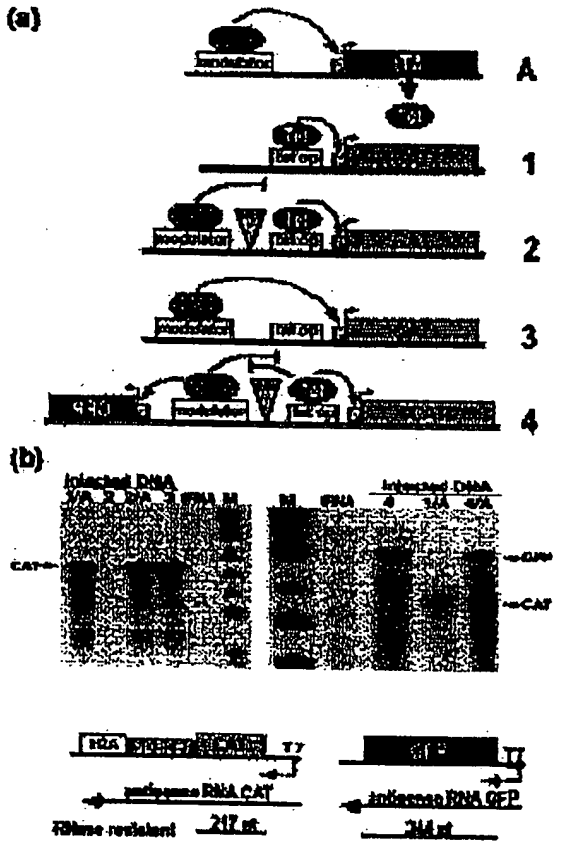

Figure 2. Polar and directional effects of *sns* on enhancer-promoter interaction. (a) Schematic representation of the microinjected plasmids. Binding of the MBF and the tTA transactivators, respectively, to the modulator and *tet* operator arrays is also indicated. Curved arrows refer to the activation of promoter by the bound factor; small arrows point to the transcription start site. (b) Total RNAs from microinjected embryos were processed to detect enhancer-activated transgene expression by RNase protection using antisense ^{32}P-labelled probes transcribed *in vitro* from the constructs illustrated below the gel images. Arrows point to the 217 nucleotide CAT and 344 nucleotide GFP RNase-resistant fragments. tRNA was used as negative RNA control. The RNase digestion products and end labelled *Hpa*II-digested pBluescript DNA (M) were run on denaturing polyacrylamide gels. Coinjection of constructs 1 and A (lanes 1/A) leads to CAT expression. In the absence of tTA, construct 2 is silent because the modulator is blocked by *sns* (lane 2). Coinjection of constructs 2 and A (lane 2/A) *trans*-activates *CAT* gene expression. Construct 3 expresses the *CAT* transgene because the *tet* operator does not interfere with the modulator (lane 3). Construct 4, in the absence of tTA, expresses only the GFP transgene (lane 4); if coinjected with A both the *CAT* and the *GFP* reporter genes are *trans*-activated (lane 4/A). Hence, *sns* does not block the binding of the transcription factors to the enhancers.

operator (construct 1). We predicted that expression of the tTA would elicit *trans*-activation of the transgene. As shown in Figure 2(b) (lane 1/A) this was indeed the case. Next, we tested the constructs with two enhancers. As expected, construct 2 containing *sns* between the MBF-1 and tTA binding sites was transcriptionally silent (lane 2), indicating that *sns* blocked the *trans*-activating function of the MBF-1 and that the *tet* operator was inactive in the absence of tTA. When the activator expression plasmid (construct A) was coinjected with construct 2, *trans*-activation of the transgene occurred (lane 2/A). The intensity of the CAT mRNA band detected in embryos injected with the two different plasmid combinations was almost identical (compare lane 1/A and 2/A), suggesting that neither the modulator nor *sns* sequences affected the extent of activation by tTA. In addition, the *tet* operator sequences did not interfere with the enhancer activity of the modulator, as similar levels of transgene expression were detected in embryos injected with construct 3 (lane 3). In summary, these experiments strongly suggest that *sns*, like chromatin insulators, has the ability to block the distal enhancer from communicating with the promoter but has no influence on the proximal one, when situated between the two.

We also investigated *sns* behavior in the context of a bidirectional transcription construct. A construct was made in which the modulator array and *tet* operator direct expression of two divergently transcribed reporter genes, encoding either CAT or green fluorescent protein (GFP). The *sns* sequence was inserted between the modulator and *tet* operator (construct 4 in Figure 2(a)). RNase protection assays were performed with RNA extracted from transgenic embryos, utilizing probes for both CAT and GFP in the same hybridization reaction. Only *CAT* mRNA was detected in embryos microinjected with constructs 1 and A (Figure 2(b), right panel, lane 1/A). As expected, in the absence of the tTA activator, the *CAT* transgene was not expressed in embryos injected with the bidirectional transcription unit (lane 4), presumably because *sns* interrupted the interactions between MBF-1 and the basal transcription apparatus. However, *sns* did not restrain MBF-1 from activating the divergent *GFP* transcription unit (lane 4). Subsequently, expression of tTA allowed for *trans*-activation of the *CAT* transcription unit (lane 4/A). From these results we conclude that *sns* blocks enhancer activity in a directional manner.

In vitro binding of proteins to *sns* sequences

To identify nuclear protein binding sites within *sns*, we performed DNase I footprint analysis and electrophoretic mobility shift assays (EMSA) with nuclear extracts from gastrula stage embryos. Two DNase I protected regions, defined as Box A and Box B, were mapped to both strands in the 5′ half of *sns* (Figure 3). The specificity of protein-DNA interaction was assessed by oligonucleotide compe-

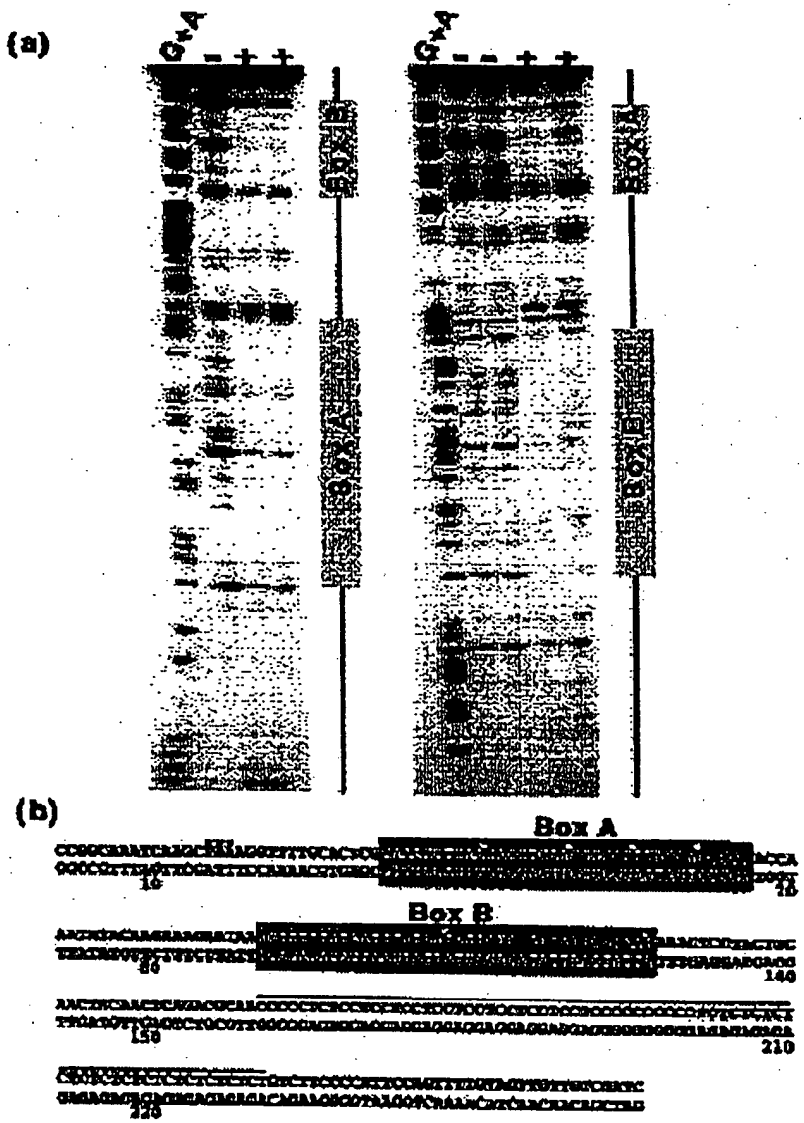

Figure 3. DNase I footprinting of an end-labelled *sns* sub-fragment spanning from nucleotides 2 to 157. (a) Sense strand (left) and antisense strand (right) were incubated with either BSA (lanes-) or nuclear extracts from gastrula stage embryos and digested with 5 μg of DNase I for three to five minutes on ice (lanes+). Digestion products were analyzed together with the cleavage products of the G + A sequence reaction on denaturing polyacrylamide gels. (b) Nucleotide sequence of the *sns* element. Asterisks mark the stop codon of the H2A gene. Sequences of protected Box A and Box B are in black boxes. Arrows inside boxes point to the Box A inverted and direct repeats and to the Box B direct repeat. Pyrimidine stretches are overlined.

tition experiments in EMSA. Figure 4 shows that both DNA-protein complexes were suppressed by an excess of unlabelled homologous probe, while they were not affected by an excess of unlabelled heterologous sequences. As indicated in the sequence shown in Figure 3(b) and in the drawing of Figure 4, Box A contains two notable sequence features: a C + A perfect direct repeat (DR), and immediately downstream the palindrome (IR) which is one of the *cis*-acting elements involved in 3′ RNA processing.[30] Because the IR sequence alone competed as efficiently as the entire Box A, we conclude that the palindrome is the protein binding site within Box A.

To search for further protein binding sites, we analyzed the pyrimidine stretch (C + T) at the 3′ end of *sns* sequences. This fragment contains 14 TC repeats that in the bottom strand correspond to seven GAGA sequences. EMSA analysis with nuclear extracts demonstrated that the C + T rich fragment formed a predominant DNA-protein complex that was specifically competed by an

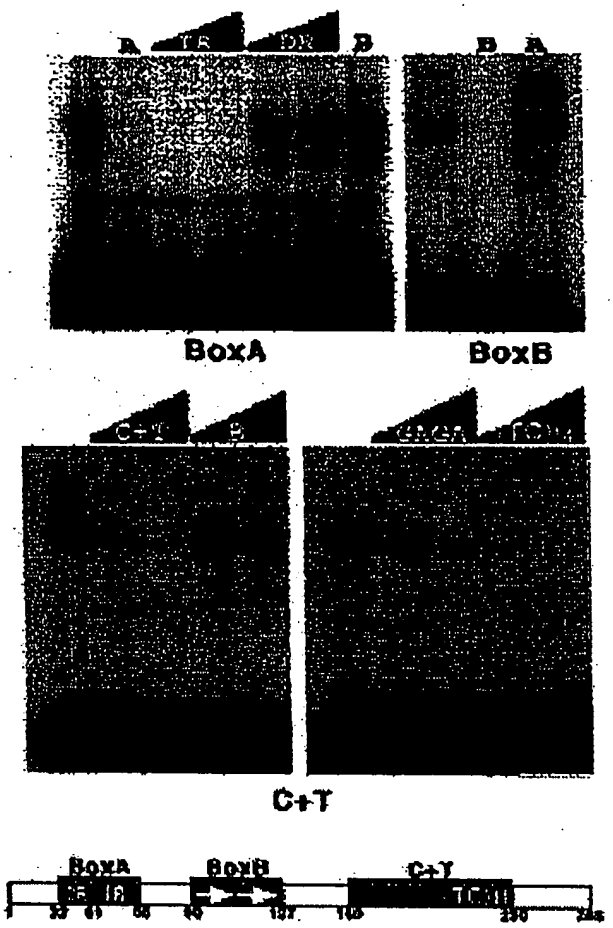

Figure 4. EMSA analysis of nuclear protein binding sites within *sns*. The three end labelled probes Box A, Box B and C+T, are underlined in the schematic drawing of the *sns* fragment. DR and IR refer, respectively, to the direct repeat and palindrome of Box A; white arrows in the Box B indicate a tandem repetition; (TC)14 refers to the 14 repetitions of the TC doublet. The C+T fragment was obtained by PCR amplification. All other probes were obtained by annealing complementary oligonucleotides. In competition experiments, nuclear extracts were pre-incubated with an excess of unlabelled homologous or heterologous probes prior to the addition of 1 ng of the labelled probe. The amounts used were: 100 ng for Box A (A) and Box B (B); 50 ng and 100 ng for IR, DR, C+T, GAGA (the GA repeats located upstream the H2A modulator), and (TC)14. The DNA-protein complexes were resolved by polyacrylamide gel electrophoresis.

excess of the homologous fragment (Figure 4). Of particular significance, protein binding was also specifically competed when nuclear extracts were pre-incubated either with an excess of a sequence containing four GAGA repeats, which is located upstream of the *H2A* modulator (see Figure 1), or with an oligonucleotide containing the 14 TC dinucleotides found at the 3′ end of the pyrimidine region (see sequence in Figure 3). The former competition was slightly less efficient, perhaps due to the presence of fewer (eight) TC dinucleotide repeats. These observations demonstrate the binding of nuclear protein(s) to GAGA sequences in sea urchin and suggest that a putative GAGA factor might contribute to the enhancer blocking function of *sns*.

Deletion of either the Box A palindrome or the 3′ CT repeats abolishes *sns* insulator function

We used the enhancer blocking assay to test the effect of 5′ and 3′ deletions of the *sns* fragment on the expression of a transgene driven by the *H2A* modulator in transgenic sea urchin embryos. The *sns* deletion mutants shown in Figure 5(a) were cloned between multiple copies of the 30 bp modulator/enhancer of the H2A histone gene and the tk promoter of the M30-CAT reporter plasmid (Figure 5(b)). Resulting constructs were microinjected into sea urchin eggs, embryos raised till gastrula stages and processed to determine *CAT* transgene expression by RNase protection analysis. Results depicted in Figure 5(c) are representative of several microinjection experiments. In agreement with our previous reports,[23,31] in the presence of one or several copies of the 30 bp histone H2A modulator sequence, transcriptional activation from the tk promoter occurs efficiently, as evidenced by abundant transgene transcripts (Figure 5(c), lanes 3, 9, 11). These M30-CAT constructs demonstrated once again the enhancer blocking function of the intact *sns* (lane 5). Deletions from either the 5′ or the 3′ that remove Box A(Δ sns), or the pyrimidine rich sequence (ΔIII sns), respectively, impaired the blocking activity of *sns* (lanes 4 and 13). In fact, levels of CAT transcripts were comparable to the construct lacking *sns* (lanes 3 and 11). As expected, 5′ deletions that

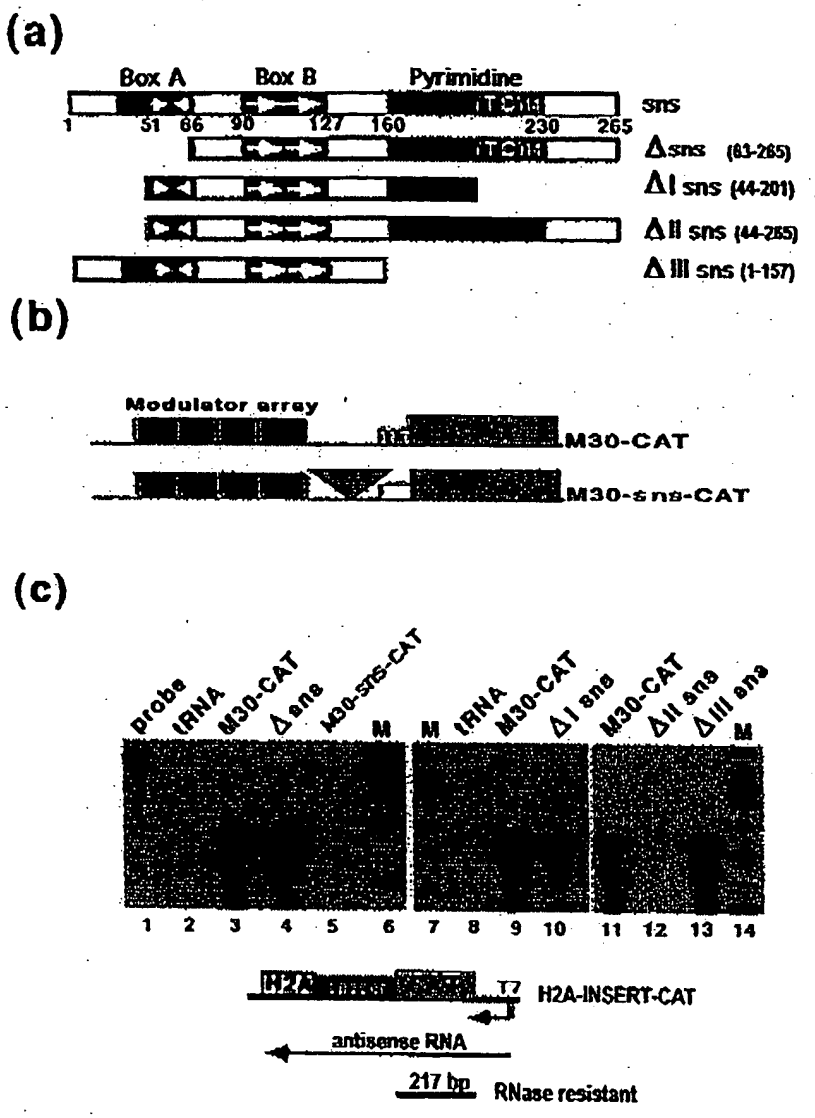

Figure 5. Functional activity of the *sns* deletion fragments. (a) Maps of *sns* and of the different deletion fragments assayed for enhancer blocking activity. (b) Schematic drawing of the microinjected plasmids. *sns* or the *sns* deletion fragments were inserted between the H2A modulator array and the tk promoter of M30-CAT to generate the M30-sns-CAT plasmids. (c) A ^{32}P-labelled antisense CAT RNA (lane 1), transcribed *in vitro* from the H2A-INSERT-CAT was used to perform RNase protection assays on microinjected embryos at gastrula stage. Electrophoretic analysis of the RNase digestion products was carried out on denaturing polyacrylamide gels. Lanes 3, 9 and 11: microinjection of the positive control M30-CAT to monitor enhancer-activated expression of the transgene. Lane 5: microinjection of M30-sns-CAT to monitor enhancer blocking activity of *sns*. Lanes 4, 10, 12 and 13: microinjection of reporter plasmids carrying, respectively, Δsns, ΔI sns, ΔII sns and ΔIII sns; only ΔII sns maintained the ability to attenuate the enhancer. Lanes 2 and 8: tRNA negative control. Lanes 6, 7 and 14: pBluescript *Hpa*II-digested end-labelled DNA markers.

left the palindrome intact, (ΔII sns) exhibited wild-type *sns* enhancer blocking activity (lane 12). Finally, removal of the TC repeats (ΔI sns) from ΔII *sns*, abolished the ability of *sns* to affect enhancer-promoter interaction (lane 10). Altogether, these results are consistent with the nuclear protein binding sites defined above and indicate that the Box A palindrome and the GAGA sites are essential for *sns* to block communication between the modulator and the tk promoter.

Box B is also essential for enhancer blocking activity

The experiments described in the previous sections suggest that the enhancer blocking function of *sns* relies on the assembly of protein complexes

at the Box A palindrome and at the GAGA sites. Because binding of proteins to Box B was also detected, we investigated whether these interactions were also essential for *sns* activity. Toward this end, we performed an *in vivo* competition experiment. We have previously used this approach to demonstrate that binding of the MBF-1 transcription factor to the modulator is required for activation of a transgene driven by the histone H2A promoter.[31] As indicated in Figure 6, sea urchin embryos were injected with the *sns*-containing transgene construct together with increasing amounts of ligated oligonucleotides containing either Box B (lanes 3, 4) or the Box A (lane 6) sequences. As levels of enhancer-activated transgene transcripts were similar to those seen with M30-CAT plasmid (lane 5), these results demonstrate that either oligonucleotide prevented enhancer blocking (lanes 2,7). Hence, titration of either Box A or Box B binding proteins by injecting their target sites impaired the ability of *sns* to block enhancer-promoter interaction.

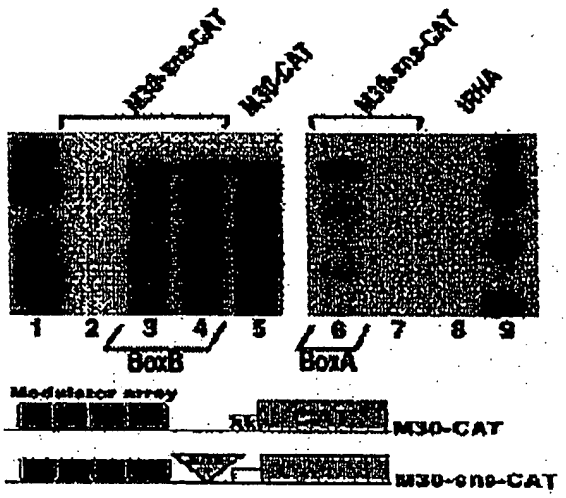

Figure 6. *In vivo* competition of *sns* function. Transgenic embryos were obtained by microinjecting the constructs drawn below the fluorograph with or without an excess of either BoxB or BoxA oligonucleotides. Black boxes represent the modulator array, the large shaded triangle the *sns* fragment. RNase protection experiments were carried out with total RNA as described in the legend to Figure 5. Lanes 2 and 7: injection of M30-sns-CAT; the enhancer is blocked. Lanes 3, 4 and 6: co-injection of M30-sns-CAT and 40-fold (lane 3) or 70-fold (lane 4) excess of ligated BoxB oligonucleotide or 70-fold (lane 6) excess of ligated BoxA oligonucleotide relieves the block. Lane 5: injection of M30-CAT; enhancer activity of the modulator array. Lanes 1 and 9 show relevant bands of DNA markers.

Discussion

Insulators are a new class of genetic elements that can modulate the activity of enhancer or other regulative sequences.[3,5] The few elements identified principally in *Drosophila* and chicken display two important characteristics: polarity and directionality of the effects of insulation of enhancer activity.[1,2] The former signifies that only enhancers located distally from the promoter with respect to the site of insertion of the insulator are attenuated in the interaction with the promoter. The second feature is that insulators do not prevent a blocked enhancer from activating transcription from a divergent promoter.[32,33] Consistently, we have shown that *sns* when placed between two enhancers, insulated the promoter-distal modulator without affecting the function of the downstream *tet* operator. In addition, *sns* did not interfere with the *trans*-activating capacity of the modulator in the other direction. Taken together, these results rule out that insertion of *sns* between enhancer and promoter represses enhancer-promoter interaction by enhancer inactivation, for example by inducing local assembly of a repressive chromatin structure.

As first shown in *Drosophila*, the directional enhancer blocking activity of insulator elements depends on the assembly of specific DNA-protein complexes. The gypsy insulator is perhaps the best-studied system with respect to the characterization of protein components that interact with insulator DNA. One of these components, the suppressor of Hairy-wing [su(Hw)] protein, binds to a reiterated target sequence[34] and recruits the second component, the mod(mdg4) protein[35] that displays properties characteristic of trithorax-group (trxG) genes.[36] The BEAF protein binds to the scs' insulator[37] which characterizes a class of chromosomal elements found at many loci.[38] Interestingly, seven tandem copies of an oligonucleotide containing BEAF binding sites has partial enhancer blocking activity.[37] The capability of the chicken HS4 insulator to interfere with enhancer-promoter interaction resides in a 42 bp fragment that contains a binding site for the CTCF transcription factor.[39] Binding of CTCF occurs also to several vertebrate insulators and to the unmethylated ICR (imprinting-control region) that displays enhancer blocking activity to control imprinted expression of the Igf2 gene.[40,41] Therefore, it is not surprising that the directional enhancer blocking activity of *sns* depends on specific DNA-protein interactions. It is of some interest that, while the enhancer blocking capacity of the *gypsy*, *HS4*, and to some extent the *scs'*, insulators relies on the recognition of a single or a reiterated binding site, *sns* contains three different *cis*-acting elements. Our results strongly suggest that all of these are needed to prevent enhancer-promoter interaction. In fact, deletion of either the Box A palindrome or the 14 TC repeats completely impaired *sns* function. Furthermore, microinjection of excess Box A or Box B and very recently GAGA (not shown) binding sites relieved the inhibition of the modulator in the *sns*-containing constructs. The most plausible explanation of the *in vivo* competition results is that the excess of

binding sites titrated, either directly or indirectly, the factors responsible for the enhancer blocking activity. Based on these observations, we speculate that *sns* achieves directional enhancer blocking activity by cooperative interactions between all three different DNA binding proteins or protein complexes.

Our results demonstrate that, within Box A, only the palindrome is required for enhancer blocking activity. Deletion of the 5′ most direct repeats, upstream of the palindrome, does not impair *sns* function. In agreement with this observation, oligonucleotides containing the direct repeats failed to compete for binding of factors to Box A and did not form specific protein-DNA complexes (not shown). Of some interest, the palindrome forms a stem-loop RNA structure, highly conserved among the non-polyadenylated histone mRNAs, from sea urchin, and *Drosophila* to mammals and represents one of the signals recognized by 3′ pre-histone mRNA processing machinery.[30]

A second *cis*-acting element was identified within the pyrimidine tract that contains seven GAGA repeats in the inverted orientation. Based upon EMSA analysis, specific protein interactions occur at the GAGA sites of *sns* and presumably at GAGA sites located upstream of the H2A modulator. Because the enhancer blocking activity of *sns* is independent of orientation,[28] it is reasonable to assume that protein(s) related to a *Drosophila* factor which binds GAGA sequences might be involved in the mechanism that interrupts the interaction between enhancer and promoter in sea urchin. *Drosophila* GAGA factor is a DNA binding protein involved in chromatin remodelling processes.[42] GAGA factor alleviates, in combination with NURF, the repressive effect of chromatin[43] and participates in the assembly of the silencing Polycomb proteins at PRE.[44] Interestingly, binding of factors to GAGA sites occurs in the spacer between the *Drosophila* H3 and H4 histone genes,[45] and recent evidence indicates a direct involvement of GAGA factor in insulator activity. GAGA factor binding sites, found at the PRE adjacent to the Fab-7 insulator, cooperate with Fab-7 to maintain the specific parasegment domain of expression of the Abdominal-B gene.[46,47] In addition, mutation of GAGA sequences within the insulator of the even-skipped locus affects directional blocking of the iab-5 enhancer.[48] Despite the similarity of the binding site and the apparent involvement in insulator function, the sea urchin protein differs from the *Drosophila* GAGA factor because a *Drosophila* polyclonal anti-GAGA factor antibody failed to supershift the *in vitro* assembled nuclear protein-DNA complex from sea urchin (not shown). The cloning of the sea urchin GAGA factor encoding gene should clarify whether the *Drosophila* and sea urchin factors are evolutionary and functionally related. One working hypothesis, currently under investigation, is that interactions between the proteins of *sns* and the proteins bound to the GAGA sites of the H2A promoter, prevent the H2A enhancer from acting promiscuously to activate transcription of heterologous early histone promoters.

With the exception of the GAGA element, the *sns* insulator sequence motifs are distinct from those described for other insulators. However, there is some evidence to suggest that these insulator sequences and their binding factors are also evolutionarily conserved. Very similar sequences are present in equivalent positions in the histone H2A transcription unit of the sea urchin *Psammechinus miliaris* (not shown). In addition, we have recently found that *sns* can insulate a viral enhancer upon stable integration in human chromatin (unpublished) and that at least two of the identified *cis*-acting insulator sequence elements, Box B and TC dinucleotide repeats (Box A did not show DNA binding activity in our conditions), interact specifically with human nuclear proteins of two different cell types (unpublished results).

In conclusion, we have extended our previous characterization of the *sns* element by the demonstration that *sns* acts equivalently to previously well-characterized insulators in a number of ways. We have now identified *cis*-acting sequences required for directional enhancer blocking activity, which may be evolutionarily conserved, and include novel sequences. Our studies have significant implications both for the control of early histone gene regulation in sea urchins, and for more general mechanisms of insulator action. In addition, these sequences may prove to have practical applicability in genetic engineering situations where insulator action might be beneficial.

Materials and Methods

Construction of plasmids

Plasmids, schematically drawn in Figure 2, were obtained as follows. Plasmid A that expresses the tTA activator, was constructed by the substitution of the CMV promoter of the pUHD 15.1 vector[29] with a fragment containing an array of the modulator sequences and the *tk* promoter. The pUHD 15.1 plasmid was digested with *Xho*I and *Xba*I simultaneously, filled in and ligated with a blunt ended DNA fragment containing the modulator sequences. Plasmid 1 that expresses the *CAT* gene under the control of the *tet* operator and the CMV promoter, was constructed by cloning the *tet* operator and the CMV promoter from the pUHD 10.3 plasmid[49] into the *Xho*I restriction site of the pBL-CAT3 vector.[50] Plasmid 3 that expresses the *CAT* gene under the control of two enhancers, the *tet* operator and the modulator, was obtained by cloning the 180 bp *Hin*dIII-*Xba*I DNA fragment containing the modulator repeats, derived from M30-CAT, in the *Hin*dIII-*Xba*I-digested plasmid 1. In construct 2, the *Hin*dIII-*Xba*I DNA fragment containing *sns* was cloned into plasmid 3 between *tet* operator and the modulator sequences. The EGFP gene (Clontech) fused to the *tk* promoter was cloned in inverted orientation upstream the modulator of plasmid 2, to generate the construct containing the two divergent transcription units (plasmid 4). To generate the plasmid M30-CAT an array of the H2A modulator/enhancer sequences was cloned into the *Sal*I site upstream of the

tk promoter of the pBL-CAT3 vector. To obtain the plasmid M30-sns-CAT, *sns* was inserted into the *Xba*I site of M30-CAT, between the modulator and the *tk* promoter. The *sns* fragment was isolated by *Hpa*II and *Sau*3A restriction enzyme digestion of the *Sac*I subclone of the PH70 histone DNA.[21] The Δsns, ΔI sns, ΔII sns and ΔIII sns deletion fragments were obtained from *sns* by restriction enzyme digestion or PCR amplification. These fragments were cloned respectively in the *Xba*I and *Bam*HI restriction sites located in the polylinker between the modulator and the tk promoter of the M30-CAT. The plasmids used for the RNase protection experiments, H2A-INSERT-CAT and GFP, were obtained as follows. The former contains a 258 bp pUC fragment inserted between the H2A promoter and a CAT coding subregion as described.[23] The latter bears a 344 bp fragment of the EGFP gene.

DNase I protection and electrophoretic mobility shift assays

Sense and antisense *sns* end-labelled fragments, spanning the region between nucleotides 2 and 157, were obtained by PCR. BSA or nuclear extracts from *P. lividus* embryos, prepared as described,[51] were preincubated with 10 μg of poly(dA-dT)·(dA-dT) in 50 μl of 10 mM Hepes (pH 7.9), 60 mM KCl, 1 mM DTT, 1 mM EDTA, 8% (v/v) glycerol for five minutes before the addition of the labelled fragments. After 20 minutes incubation, samples were digested with 5 μg of DNase I in 2.5 mM $MgCl_2$ for three to five minutes at 4°C, phenol extracted and loaded on 8% (w/v) denaturing polyacrylamide gels. In EMSA experiments, three different *sns* probes were assayed for binding activity *in vitro*: Box A, Box B double-stranded oligonucleotides (see next paragraph for sequences) and the C + T containing subfragment (spanning nucleotides 137 and 262) obtained by PCR. 1 ng of end labelled Box A , Box B and C + T probes were incubated, for 30 minutes on ice, with 5 μg of nuclear extracts from sea urchin embryos at gastrula stage and 2 μg of poly(dI-dC)·(dI-dC) in 20 μl of 10 mM Hepes (pH 7.9), 60 mM KCl, 1 mM DTT, 1 mM EDTA, 4% Ficoll. In the competition experiments, unlabelled homologous or heterologous probes in the amounts described in the legend to Figure 4 were added to the preincubation mixture prior to the addition of the extracts. Binding reactions were run on non-denaturing polyacrylamide gels in 50 mM Tris, 50 mM H_3BO_3 and 2 mM EDTA (pH 8.3).

List of oligonucleotides

Only the sense strand is reported. Residues of the following oligos refer to the first nucleotide of the *sns* sequence shown in Figure 3 (accession number Y09062): Box A: CAAACCTCAACACCTCAACGGCCCTTATCAGGGCCACCA (spanning nucleotides 32 to 70); Box B: AGTCTCTGTAATTCATAATAGTCTCTGTAATTCATAAT (spanning nucleotides 90 to 127); DR: CAAACCTCAACACCTCAAC (spanning nucleotides 32 to 51); IR: CTCAACGGCCCTTATCAGGCCCACCAA (spanning nucleotides 45 to 71); (TC)14:CCTCTCTCTCTCTCTCTCT CTCTCTCTCTCTCTGTCT (spanning nucleotides 200 to 234). The GAGA oligonucleotide, GGGAGGGAGAGAGAGAGAGAGA, spans nucleotides −200 to −177 from the transcription starting site of the H2A promoter.

Microinjection and transgene expression

Plasmids were linearized with *Hin*dIII or *Cla*I restriction enzymes whose sites are located in the polylinker regions, 5′ and 3′ to the CAT sequence, respectively. Linearized plasmids were brought to a total concentration of 240 μg/ml with threefold excess of *Hin*dIII-digested *P. lividus* sperm DNA of roughly 5 kb. Unfertilized eggs from a mature female were dejellied in pH 5.0 sea water, immobilized on Petri dishes with 1% (w/v) protamine sulfate and injected into the cytoplasm with 1-2 pl DNA in 25% glycerol solution. Microinjected eggs were fertilized and embryos raised up to the early gastrula stage (14 hours development). 3000 uninjected embryos at the same developmental stage were then added as carrier, and embryos collected by low speed centrifugation. Total RNA was extracted by one hour incubation at 55°C in a solution containing 200 μg/ml Proteinase K and phenol extraction. Nucleic acid samples were digested with RNase-free DNase I, and RNA from 50 microinjected embryos was hybridized with [^{32}P]UTP-labelled antisense CAT or EGFP RNA transcribed *in vitro* from the H2A-spacer-CAT and GFP plasmids. Hybridisation conditions, RNase digestion and gel fractionation of the RNase-resistant hybrids were as previously described.[52]

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Tetracycline-Regulated Gene Expression Switch in *Xenopus laevis*

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***Xenopus* is a well-characterized model system for the investigation of biological processes at the molecular, cellular, and developmental level. The successful application of a rapid and reliable method for transgenic approaches in *Xenopus* has led to renewed interest in this system. We have explored the applicability of tetracycline-regulated gene expression, first described by Gossen and Bujard in 1992, to the *Xenopus* system. By optimizing conditions, tetracycline repressor induced expression of a luciferase reporter gene was readily and reproducibly achieved in both the *Xenopus* oocyte and developing embryo. This high level of expression was effectively abrogated by addition of low levels of tetracycline. The significance of this newly defined system for studies of chromatin dynamics and developmental processes is discussed.** © 2000 Academic Press

Key Words: Xenopus; chromatin; transcription; development; gene expression; tetracycline.

INTRODUCTION

The *Xenopus* oocyte and embryo have provided powerful model systems for the elucidation of mechanisms governing cellular and developmental processes [1–4]. In the case of the embryo, the description and use of a rapid and reliable *Xenopus* transgenic approach [5–8] have given a new perspective to *Xenopus* developmental studies [9]. This approach permits the overexpression of gene products in every cell of the organism or in a specific tissue. With this new technology comes the need to define parameters for its effective application, including the adaptation of existing transgenic methodologies. One of the invaluable features of the *Xenopus* oocyte system is its capacity to efficiently transcribe foreign genes encoded on microinjected plasmids following their assembly into chromatin [1, 10–13]. Recent evidence has suggested that processes regulating chromatin stability are linked to the transcriptional regulatory machinery (reviewed in [14]), highlighting the need to examine nuclear processes in a chromatin context. Since both transcription and replication can be assessed on templates assembled into chromatin using the *Xenopus* system, regulatory tools for use in the *Xenopus* oocyte or embryo would have important applications for the study of interactions between chromatin and the transcription and replication machinery. One such regulatory tool is the tetracycline-mediated gene expression switch.

Tetracycline-controlled gene expression was first described by Gossen and Bujard in 1992 and utilizes the very specific and high affinity binding of the *E. coli* tetracycline repressor protein (tetR) to its operator sequence (tetO) [15]. Using a fusion protein consisting of tetR fused to the VP16 activation domain (tTA) in HeLa cells, a luciferase reporter gene was activated up to five orders of magnitude and "turned off" to basal levels by the addition of low amounts of tetracycline to the tissue culture media. This tightly regulated genetic switch has been employed in a variety of studies where conditional gene expression is required. It has been used successfully in transgenic mice [16–18], where it is particularly appealing when the gene products under study are toxic or inhibitory to embryonic development. To address more diverse questions, the effective binding of tetR to its operon sequence has been exploited for purposes other than gene regulation. For example, it has been used in yeast to mark a specific region of DNA for mapping of sister chromatid separation with a tetR/GFP fusion protein bound to an array of tetO sites [19–21]. In the context of chromatin, tetR was demonstrated to form a physical boundary to nucleosome mobility in an *in vitro Drosophila* assembly system, thereby establishing a means to functionally analyze the chromatin remodeling machine CHRAC [22]. Here we describe, for the first time, the optimal conditions for successful application of this tetracycline-regulated switch for *in vivo* approaches in both the *Xenopus* oocyte and embryo.

MATERIALS AND METHODS

Antibodies and Constructs

pUHC13.3 contains seven tetO binding sites upstream of the minimal CMV promoter driving the luciferase reporter gene and pUHD15.1 expresses tTA protein. Both were generous gifts from S. Robine and have been previously described [15]. tTA mRNA was transcribed *in vitro* from the pSP65tTA plasmid constructed by in-

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0014-4827/00 $35.00

serting the tTA coding region from pUHD15.1 into pSP65 by *Eco*R1/*Bam*H1 digestion and ligation. M13E4tetO was produced by removing the five Gal4 binding sites from M13E4G5 [2] by a *Hin*dIII/*Bam*HI digest and blunt ligation to the seven tandem repeats of tetO removed from pUHC13.3 by *Xho*I/*Stu*I digestion. The sequences and orientation of constructs were confirmed by sequencing. The TetR monoclonal antibody was raised against the tetracycline-responsive transcriptional activator tTA (Clontech catalog no. 8632-1) and was used at a 1:500 dilution for Western blotting according to standard protocols.

Xenopus Microinjection Strategy

Stage VI *Xenopus* oocytes were surgically removed and treated with collagenase as previously described ([11]; for methodological reviews, see [23]). *In vitro* transcribed tTA mRNA (quantity and quality assessed by UV analysis and electrophoresis) was injected into the cytoplasm of stage VI oocytes using a Drummond Nanoject automatic injector and incubated at 16°C overnight to allow tTA protein expression and accumulation. Approximately 18 h later, the tTA-regulated luciferase reporter pUHC13.3 [15] was injected into the nucleus and oocytes were incubated +/− 200 ng/ml tetracycline hydrochloride (Sigma) for a further 5 h to allow time for chromatin assembly on the reporter plasmid and luciferase expression. Ten healthy oocytes were recovered and lysed in 100 μl of lysis buffer, and the levels of luciferase reporter activity were assessed as described in the luciferase detection kit (Perkin–Elmer). Following lysis, DNA and RNA were analyzed as previously described [2].

Fertilized *Xenopus* eggs were coinjected into one blastomere at the 2-cell stage of development with 50 pg of pUHC13.3 reporter DNA and various amounts of tTA mRNA in a total volume of 26.7–32.2 nl as previously described [3]. Embryos were incubated +/− 2 μg/ml tetracycline hydrochloride for at least 14 h at 23°C. Alternatively, tetracycline was dissolved in water and coinjected into the embryos with pUHC13.3 and tTA mRNA to give a final concentration in the embryo of approximately 500 ng/ml. Embryos were lysed at various stages of development [24] and luciferase activity assessed.

Transcription Analysis

To assess the levels of either luciferase or E4 transcript accumulated per DNA template, a reverse transcription assay was performed as previously described [2]. Accumulation of luciferase mRNA transcript from 5 ng/oocyte pUHC13.3 luciferase reporter was detected by reverse transcription from an end-labeled oligo(5′-AGCCTTATGCAGTTGCTCTC-3′) annealed to luciferase mRNA extracted from the oocyte as previously described [2]. Extension gives rise to a product of 306 nucleotides. The same method was used to detect the E4 transcript, using in this case the end-labeled oligo(5′-CTTCACACCGGCAGCCTAACAGTCAGCC-3′), which produces a major product of 100 nucleotides. Efficiency of microinjection and chromatin assembly on transcribed templates was assessed by performing a supercoiling assay in parallel on DNA extracted from the same lysate used to prepare the RNA for reverse transcription analysis. Each lane of gel represents extension product from RNA accumulated in 2 oocytes. Quantification of transcripts and DNA recovery was performed with a PhosphoImager (STORM).

Supercoiling Assay

Following lysis of oocytes, a supercoiling assay was used as a measure of the efficiency of microinjection and assembly of injected templates into chromatin as previously described [2]. Each lane of the supercoiling assay represents DNA extracted from 5 oocytes from an injection of 5 ng/oocyte. Following electrophoresis, the DNA was Southern blotted and hybridized to M13E4tetO radioactively labeled with ^{32}P by random priming (Amersham Rediprime II RPN 1633).

RESULTS

Tetracycline-Regulated Gene Expression in the Xenopus Oocyte

It has been reported that the responsiveness of tetracycline-inducible systems can vary, depending on the cell type [25, 26]. In order to determine whether conditional gene expression could be achieved in *Xenopus* using the tetracycline-regulated system, we first asked whether expression of a luciferase reporter could be induced by tTA using a transient assay. The tTA protein was produced in the oocyte following cytoplasmic microinjection of *in vitro* transcribed tTA mRNA. This approach has previously been reported to be a reliable method for introducing foreign proteins into the oocyte [27–29], where the activity of injected RNA may be further improved by flanking the RNA with untranslated regions of the *Xenopus* globin gene. Therefore, we determined the activation threshold using 5 ng of microinjected reporter DNA when coinjecting increasing amounts of tTA mRNA. First, we confirmed that tTA protein was being produced in the oocyte at levels corresponding to the increase in injected mRNA (Fig. 1A). Second, we measured accumulation of luciferase mRNA by a reverse transcription assay (Fig. 1B). When the level of luciferase transcript was assessed, it was evident that an increase in activation of transcription from this reporter could be obtained using up to 75 ng of tTA mRNA, with 5 ng of reporter DNA reaching an optimal induction between 60 and 75 ng (Fig. 1B). The amount of tTA mRNA required to reach this optimal level is within the previously reported limit of 100 ng of mRNA that can be expressed by a single oocyte [30]. Factors available for luciferase protein expression also appear to be limiting since luciferase activity reaches a peak at 45 ng of injected tTA RNA (Fig. 1C), indicating that when high quantities of DNA are required, transcription efficiency should be assessed by analysis of the resulting transcript rather than the protein product.

Luciferase activity from the pUHC13.3 reporter was subsequently used as a measure of tTA-regulated expression of low levels of reporter since it has been well characterized for transient assays in tissue culture cells [15, 31, 32]. Figure 2A illustrates the experimental strategy taken for introduction of the tTA protein and luciferase reporter and analysis of products in the *Xenopus* oocyte. With this strategy, a range of amounts of both reporter plasmid and tTA mRNA gave high levels of reporter induction (Fig. 2B), achieving almost 50-fold induction of luciferase activity above basal levels expressed from the reporter without the tTA activator. An optimal induction was reached when 250 pg of reporter and 15–22 ng of tTA mRNA were microinjected since no significant increase in activation was

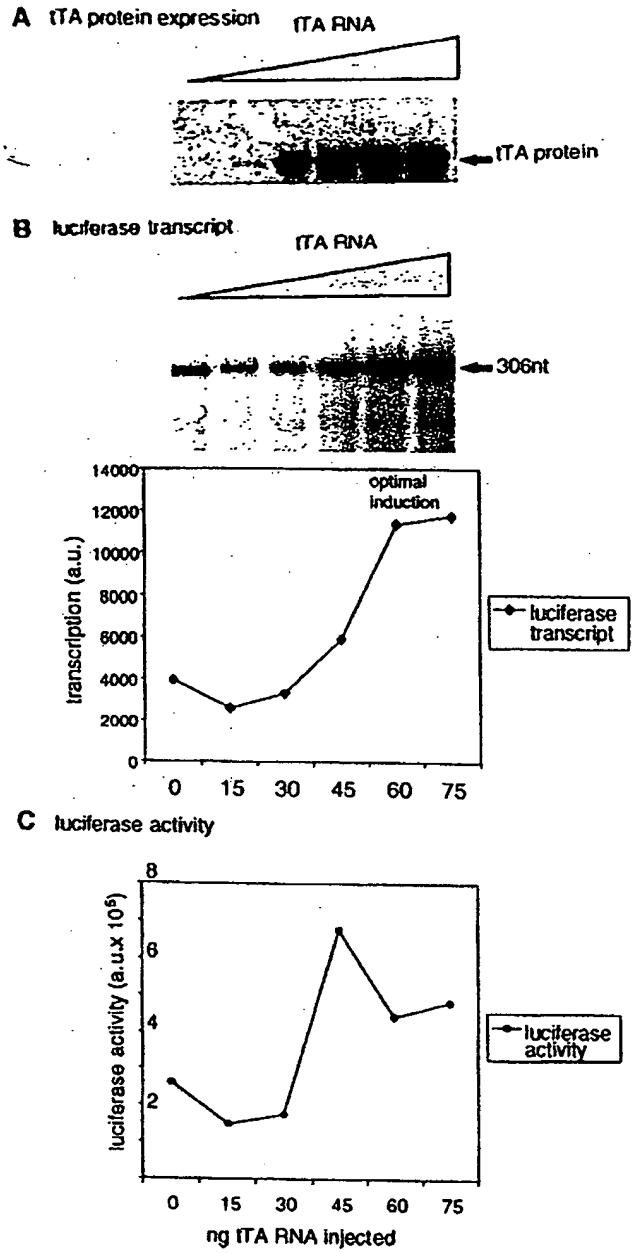

FIG. 1. Optimizing conditions for microinjection of tTA mRNA in the *Xenopus* oocyte. (A) Western analysis of *in vivo* translated tTA protein from oocyte extracts. Stage VI *Xenopus* oocytes were injected into the cytoplasm with the following increasing amounts of *in vitro* transcribed tTA mRNA: 0, 15, 30, 45, 60, and 75 ng/oocyte. Following injection, oocytes were incubated overnight for tTA protein expression and accumulation. Fifty oocytes were homogenized for each variable and centrifuged at 35,000 rpm and the clear protein extract layer was removed. Extract from the equivalent of 2 oocytes was then analyzed by Western blotting and revealed using a TetR monoclonal antibody (Clontech catalog no. 8632-1) at a 1:500 dilution and chemiluminescence according to manufacturer's instructions (Pierce SuperSignal, catalog no. 34080). (B) tTA-activated transcription from the pUHC13.3 reporter. (upper panel) Transcription of luciferase mRNA from 5 ng of pUHC13.3 luciferase reporter was detected by reverse transcription as described under Materials and Methods. The expected size of the reverse transcription product is given at 306 nt. A range of tTA mRNA of 0, 15, 30, 45, 60, and 75 ng/oocyte was injected in each case. (lower panel) Graphic illustration of accumulated luciferase mRNA, as described above, analyzed by phosphoimaging of the upper panel reverse transcription reaction. Optimal induction is indicated. (C) tTA-activated luciferase activity. Expression of luciferase from 5 ng of pUHC13.3 luciferase reporter was measured by a luciferase assay as described under Materials and Methods. A range of amounts of tTA mRNA of 0, 15, 30, 45, 60, and 75 ng/oocyte was injected as indicated.

detected despite the increased tTA RNA injected. This suggests that at this level all tTA binding sites are occupied. Significantly, addition of 200 ng/ml of tetracycline to the culture media was sufficient to completely abrogate this induction (Fig. 2B). Therefore, low levels of injected reporter DNA produced conditional gene expression, illustrating the effectiveness of this regulatory system in the *Xenopus* oocyte.

Tetracycline-Regulated Gene Expression during Development

Because of the high specificity and the low toxicity of the tetR protein and the tetracycline effector [15, 16], we wanted to determine the effectiveness of this system for use in the developing *Xenopus* embryo. Using an experimental strategy depicted in Fig. 3A, we coinjected fertilized eggs with luciferase reporter and various amounts of tTA mRNA to define the optimal parameters for tTA-driven promoter activation. Levels of injected DNA were kept to a minimum (50 pg/embryo) since higher amounts of DNA injected during early development result in low survival rates [33]. The most effective level of tTA mRNA to coinject with 50 pg of reporter was assessed at two different stages of development and determined to be 15–22 ng/embryo (data not shown). With these optimal conditions, high and reproducible levels of conditional gene expression were achieved in the developing embryo (Fig. 3B) following the midblastula transition when zygotic transcription is initiated (reviewed in [34]). At stage 11, although there is background luciferase activity expressed from the reporter plasmid, indicating that the embryos have developed beyond the MBT, there is no evidence of induction of luciferase expression by tTA, suggesting that not enough time has elapsed to establish levels of tTA protein necessary for activation of transcription. By stage 12, however, there is an effective induction of luciferase expression by tTA to over 100-fold, which is maintained through early development, with the peak of activation at stage 19 producing over 200-fold stimulation above levels with the reporter alone. By stage 27, the level of activation has dropped to 74-fold, indicating that the maximal limits of the system may have been reached by this stage. Significantly, levels of injected DNA are reported to decline following gastrulation [35], which may reflect the inability of even high levels of injected

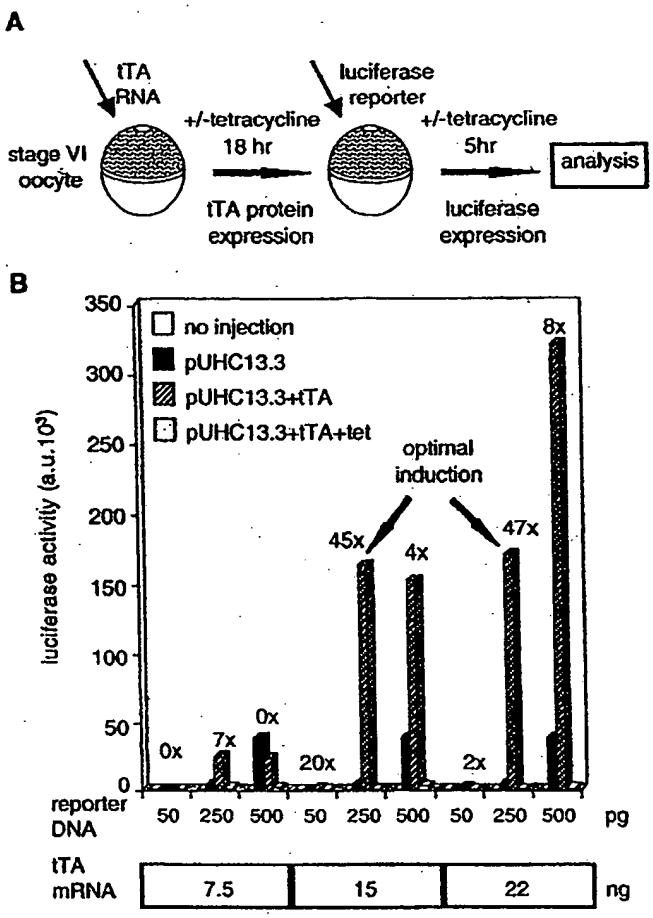

FIG. 2. Tetracycline-regulated gene expression in the *Xenopus* oocyte. (A) Experimental strategy for the tetracycline-regulated gene expression system in the *Xenopus* oocyte. Stage VI *Xenopus* oocytes were surgically removed and treated with collagenase as previously described [11, 23]. *In vitro* transcribed tTA mRNA was injected into the cytoplasm of oocytes, which were subsequently incubated at 16°C overnight +/− 200 ng/ml tetracycline hydrochloride (Sigma) to allow tTA protein expression and accumulation. Approximately 18 h later, the tTA-regulated luciferase reporter pUHC13.3 [15] was injected into the nucleus of the same oocytes and incubation continued +/− tetracycline for a further 5 h to allow time for chromatin assembly on the reporter plasmid and luciferase expression. (B) tTA induction of luciferase activity in *Xenopus* oocytes. Histograms illustrate the levels of luciferase activity, in relative light units, detected in the equivalent of 2 oocytes from the lysis of a total of 10 healthy oocytes per variable. Levels of induction and its abrogation on addition of 200 ng/ml tetracycline are shown after injecting 50, 250, or 500 pg of pUHC13.3 luciferase reporter and either 7.5, 15, or 22 ng of tTA mRNA/oocyte as indicated. Optimal induction and fold activation +tTA are indicated.

tTA mRNA to increase luciferase expression at this time. The above results indicate that the optimal induction of gene expression driven by the tTA protein using this transient assay is obtained at stage 19 of development, with high conditional activation achieved from stages 12 through to at least 27 using a ratio of 1:440 coinjected reporter DNA to tTA mRNA. We confirmed that the injected mRNA was translated into tTA protein by Western blotting of embryo extracts and that this level of mRNA was not inhibitory to early embryonic development (Fig. 3C). Injected DNA has been shown to persist for many months at low levels in the developing *Xenopus* [33, 35] probably following its integration into the host cell genome, suggesting that a mosaic pattern of induction may be achievable even at advanced stages of development using the protocol outlined in Fig. 3A. In fact, we were able to detect some activity from the luciferase reporter to the swimming larvae stage (stage 43) (data not shown).

Although it is clear that tTA can reach its tetO binding site to activate transcription in the embryo, results from these experiments indicate that tetracycline in the culture media has no effect on this activation (Fig. 3B, gray histograms). In a subsequent tetracycline titration experiment, a similar lack of effect was observed using 200 ng/ml and 0.02 mg/ml tetracycline (data not shown). Very high levels of tetracycline caused an arrest in development (at stage 13 for 0.2 mg/ml and stage 7 in the case of 2 mg/ml). Although the tetracycline-mediated shutoff of gene expression by tTA can be effectively achieved in tissue culture cells [15, 30] and in the *Xenopus* oocyte (Fig. 2B), it is not as rapidly established in whole organisms. Tetracycline can reduce levels of tTA-induced expression in transgenic mice implanted with slow-release tetracycline pellets, but this is often measured following 7 days of implantation [16] and variations in effectiveness with tissue type have been shown to exist [16–18]. We reasoned that *Xenopus* embryos could be more resistant than oocytes to the diffusion of tetracycline from the culture media. To address this question, we coinjected tetracycline with tTA mRNA and reporter DNA rather than adding tetracycline to the culture media. With this protocol, tTA-induced activation of luciferase was abolished by tetracycline even though injection of tetracycline with the reporter alone did not change the basal levels of luciferase expression (Fig. 4). The elimination of luciferase activity by tetracycline injection (compare pUHC13.3 + tTA histogram to pUHC13.3 + tTA + tet histogram) is remarkable considering the lack of tetracycline-mediated shutoff detected in Fig. 3B at a similar stage of development (stage 12, gray histogram). The tetracycline-regulated release of tetR from its recognition site can thereby be achieved using this coinjection strategy; however, optimization of this parameter will be needed to fully exploit the tetracycline-regulated system in the embryo.

Chromatin Assembly Coupled to Second-Strand Synthesis Eliminates Background Expression

To regulate gene expression in either the oocyte or as an integrated gene in a transgenic frog, the tTA protein must be competent to access its binding site on a DNA

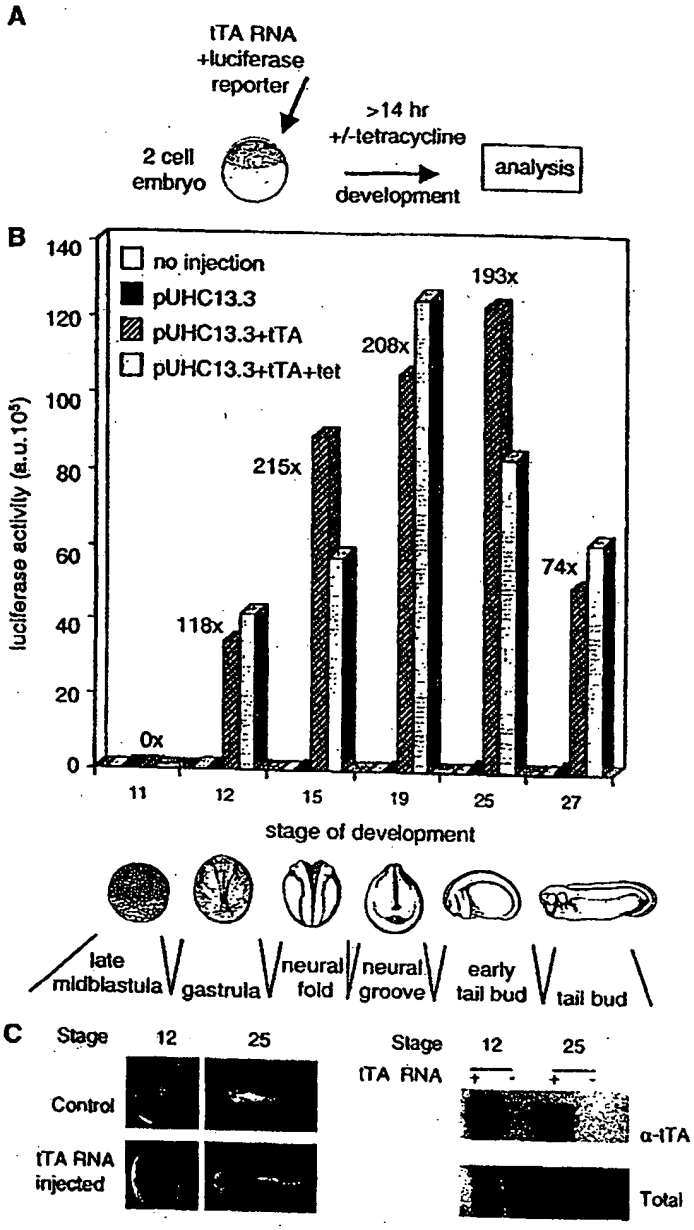

FIG. 3. Tetracycline-regulated gene expression during *Xenopus* development. (A) Experimental strategy in developing embryos. Fertilized *Xenopus* eggs were coinjected into one blastomere at the 2-cell stage of development with 50 pg of pUHC13.3 reporter DNA and various amounts of tTA mRNA in a total volume of 26.7–32.2 nl as previously described [3]. Embryos were incubated +/− tetracycline hydrochloride for at least 14 h at 23°C and lysed at various stages of development [24] to assess luciferase activity. (B) tTA induction of luciferase expression in the developing embryo. Histograms illustrate luciferase activity, in relative light units, detected from 2 embryos at various stages of development [24] as defined beneath (diagrams from Xenopus Molecular Marker Resource at http://vize222.zo.utexas.edu/). In each case, 22 ng of tTA RNA and 50 pg of pUHC13.3/embryo were coinjected and embryos incubated +/− 2 μg/ml tetracycline. Fold of activation +tTA is indicated for each developmental stage. (C) Expression of the tTA in the developing embryo. Left: pictures of control (top) and injected embryos with 25 ng of tTA RNA (bottom) were taken at the stages 12 and 25 according to [24]. Right: Western blot analysis of the corresponding embryos. The tTA protein is detected using the TetR monoclonal antibody (top panel) and the total protein is detected using Ponceau staining (bottom panel). The equivalent of 2 embryos is loaded in each lane.

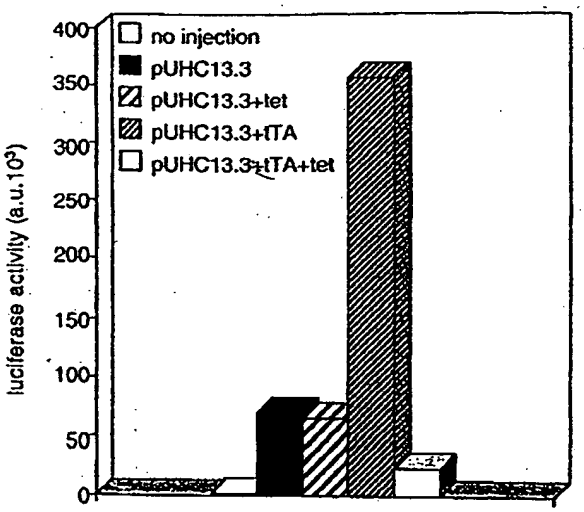

FIG. 4. Microinjection of tetracycline to regulate tTA binding. Histograms illustrate luciferase activity, in relative light units, detected from 3 embryos at stage 11–12 (midblastula) of development. In each case, 50 pg of pUHC13.3 and/or 22 ng of tTA mRNA per embryo was coinjected. Some samples, as indicated, were coinjected with a tetracycline solution to give a final concentration in the embryo of approximately 500 ng/ml.

template assembled into chromatin. Therefore, to extend the use of the induction system for analysis of microinjected reporter DNA assembled into chromatin, we further refined the conditions. Higher amounts of reporter DNA are required in this case since 1–5 ng of injected double-stranded plasmid DNA is the minimum amount necessary for effective chromatin assembly in the *Xenopus* oocyte [36]. We know from our initial experiments (Fig. 1) that 5 ng of reporter DNA is optimally activated by injection of 60–75 ng of tTA mRNA, indicating that tTA-regulated gene expression occurs on chromatinized templates, illustrating its potential as a tool either for regulation of integrated genes in transgenics or for *in vivo* chromatin studies. However, when we increase the amount of reporter DNA, background expression of luciferase is detected concomitant with a decrease in the activation potential (see Fig. 2B, 500-pg level, and Fig. 1B, basal transcription level). The high level of basal transcription from the minimal CMV promoter in this reporter construct is not surprising since genes driven by the CMV promoter are known to be highly expressed in the oocyte [2]. To eliminate this background expression from the reporter and to assess the effectiveness of this system in an integrated gene, we initiated a single-stranded (ss) template strategy. It has been shown that ss tem-

plates injected into oocytes are assembled into chromatin during the process of second-strand synthesis, which is repressive to basal transcription (initially reported in [2] and subsequently applied in [12, 29, 37, 38]). We reasoned that the introduction of the tTA-driven promoter on a ss template would provide a much more tightly regulated on/off system by repressing the effects of basal transcription. In addition, the ss template, once assembled into chromatin in the oocyte, would produce a template with the characteristics of an integrated promoter. Therefore, we constructed an M13 derivative (M13E4tetO) by removing the seven tetO binding sites from pUHC13.3 and inserting them into M13E4G5 [2] in place of the five Gal4 binding sites. Using this construct, we can compare both basal and tTA-activated transcription of the E4 gene during second-strand synthesis coupled chromatin assembly using a reverse transcription assay (Fig. 5A). When the ss template is injected, basal transcription is not detected (lane 1) whereas the double-stranded (ds) template shows background expression (lane 2) as expected. Although both the ss and ds templates were assembled into chromatin (as indicated by supercoiling, Fig. 5B), the tTA protein was able to overcome this chromatin-repressed state and activate transcription (lanes 3 and 7 for ss template and lanes 4 and 8 for ds template). Addition of 200 ng/ml of tetracycline to the oocyte culture media turned off the expression of the E4 gene (lanes 5 and 9 for ss template and lanes 6 and 10 for ds template). Therefore this ss template strategy provides a tightly regulated system to examine questions related to transcription from chromatin templates in the oocyte. It also indicates that the tetO binding sites would be accessible to tTA protein in an integrated promoter and that basal expression would be eliminated, providing an attractive mechanism for regulating overexpression of otherwise deleterious gene products in a transgenic *Xenopus*.

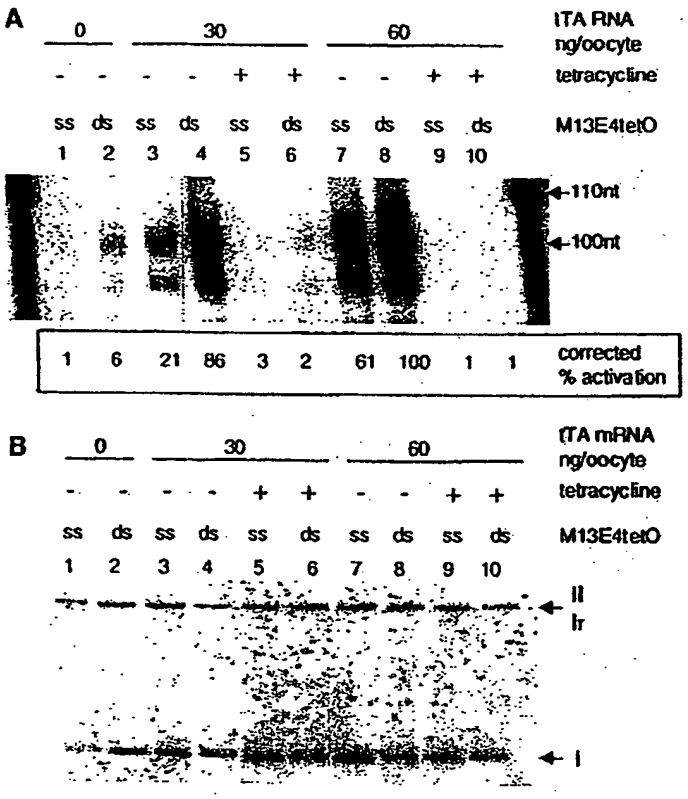

FIG. 5. Single-stranded strategy for tetracycline-regulated transcription. (A) Tetracycline-regulated E4 transcription. Transcription of the E4 gene from microinjected ss or ds M13E4tetO in *Xenopus* oocytes was detected using the reverse transcription assay described under Materials and Methods from oocyte extracts using the strategy illustrated in Fig. 2A. Either 5 ng/oocyte M13E4tetO alone (lanes 1 and 2) or in combination with 30 ng of tTA mRNA (lanes 3–6) or 60 ng of tTA mRNA (lanes 7–10) were microinjected into oocytes. Tetracycline (200 ng/ml) was added to the culture media in some cases (lanes 5, 6, 9, and 10). For each variable, 15 oocytes were lysed and the equivalent of 10 oocytes used for RNA extraction (A) and 5 oocytes for DNA extraction and supercoiling assay (B). Each lane represents mRNA extracted from 2 oocytes. The percentage activation is given for each lane corrected for amount of supercoiled template as shown in B. Marker at 110 nt is shown on right and left of gel. (B) Chromatin assembly on microinjected M13E4tetO templates. The supercoiling assay was used as a measure of chromatin assembly in the oocyte. Fifteen oocytes from each variable were pooled and lysed, with the equivalent of 10 oocytes used for RNA extraction as described above and 5 oocytes for DNA extraction and supercoiling assay. Lanes are the same as described for A. In each case, the efficiency of the microinjection was determined based on the amount of circular supercoiled DNA (I). The different forms of DNA are indicated as circular supercoiled (I), relaxed (Ir), and nicked (II).

DISCUSSION

In this report we define the optimal conditions for tetracycline-regulated gene expression in the *Xenopus* system. Information presented in Fig. 1A clearly shows that the tTA activator can be expressed to high levels in the oocyte. Importantly, the ability to assess the introduction of the tTA protein at the single-cell level in the *Xenopus* oocyte enabled us to determine that high levels of tTA (expressed from 75 ng of mRNA; Fig. 1A) can be introduced into an oocyte with no detectable deleterious effects on transcription (Fig. 1B). High levels of tTA protein can also be expressed in *Xenopus* embryos without gross developmental abnormalities (Fig. 3C). This is of interest since the tTA protein has been suggested to have toxic effects [17]. We also know that this tTA protein binds to the tetO sites since it activates luciferase expression 50-fold in the oocyte and over 200-fold in the developing embryo. The advantage of the tetR system over other activating systems such as hormone/receptor-driven systems [12, 29, 39] is the capacity to turn off activation, permitting gene activation or repression over a defined window, making it ideal for developmental applications and transgenics.

Refinements in restriction-mediated transgenic sys-

tems for *Xenopus* [5] allow overexpression of a particular gene product such as a kinase-deficient dominant-negative FGF receptor [6]. Application of tetracycline-regulated expression would improve its regulatory potential. During early stages of development, gene expression could be controlled by tetracycline injection. At later stages, when tadpoles or adults are feeding, tetracycline could be added to the food or water supply. In tetracycline-regulated mouse transgenics, a reverse rtTA induction system [18], where addition of tetracycline results in induction rather than shutoff, has overcome problems associated with the tTA system, where the half-life and clearance of the inducer are required for regulation (reviewed in [40]). This reverse system may prove to be a more effective alternative in the *Xenopus* embryo to maintain a prolonged shutoff of gene expression followed by a rapid burst of activation during a specific developmental stage or in a specific tissue. This burst of activation could be readily monitored by coupling the expression of the gene of interest to a fluorescent marker protein such as GFP.

Fluorescent markers have been used to track cellular processes by linking them to proteins and following their path during cell division or development. In such an approach, the LacI repressor fused to GFP was used to follow the localization of lactose operon operator sequences inserted into replication origin regions in *B. subtilis* [41]. An elegant series of experiments in yeast [19–21] make use of the tetracycline operator/repressor system to follow sister chromatid separation to identify factors involved in sister chromatid cohesion. The efficiency of expression and binding of tetR to its operon in *Xenopus* demonstrated in this report would facilitate the rapid application of such approaches to the study of nuclear dynamics in this higher eukaryotic system.

We are presently applying the tetracycline-regulated system to explore questions related to chromatin dynamics. Recent reports examining the action of the chromatin-remodeling machines in modifying chromatin suggest a tracking or sliding mechanism rather than the previous notion of nucleosome displacement [22, 42, 43]. In this context the binding of tetR has been used as a physical boundary to the mobility of nucleosomes during *in vitro Drosophila* chromatin assembly [22], lending further credibility to a sliding mechanism of action for CHRAC. We are using tetR as a barrier to chromatin assembly to plot the progress of bidirectional repair coupled chromatin assembly [44, 45] along DNA.

In conclusion, we have extended the application of tetracycline-regulated gene expression by defining the optimal parameters for its use in both the *Xenopus* oocyte and developing embryo. In both cases conditional expression of a gene of interest can be reliably and reproducibly achieved, adding to the already well-established usefulness of the *Xenopus* model system.

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An In Vivo Doxycycline-Controlled Expression System for Functional Studies of the Retina

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Purpose. Transgenic mice were developed that express tetracycline-controlled transactivator 1 (tTA1) specifically in photoreceptor cells. In these mice the transcription of the gene of interest can be easily inactivated in the retina in a short time frame.

Methods. A construct was prepared containing tTA1 under control of the murine rhodopsin regulatory region. This construct was used for the generation of transgenic mice. In situ hybridization was performed to study the distribution of the transactivator in the retina. The activity of the transactivator was analyzed by mating the lines with a luciferase reporter transgenic mouse. tTA1 activity and doxycycline's ability to block it were analyzed by luciferase assay. The effects of tTA1 on the retina were assessed by histology and electrophysiology.

Results. Two transgenic lines were developed that specifically express tTA1 in photoreceptor cells. The time course of transgene expression replicated transcription of endogenous rhodopsin. tTA1 was not toxic to the retina. Transactivator activity was blocked readily by doxycycline.

Conclusions. An expression system for photoreceptor cells was generated to drive transcription in a cell-specific and time-controllable manner. This system is suitable for the study of factors involved in retinal biology and of mutant forms of genes involved in retinal diseases. (*Invest Ophthalmol Vis Sci.* 2003;44:755–760) DOI:10.1167/iovs.02-0340

Functional studies of the retina would benefit from systems that allow the control of gene expression at a temporal and spatial level. For instance, the ability to block expression of either a protein or a mutant allele in a fast and complete way at a specific time point would be very useful in determining whether elimination of this factor changes either retinal physiology or a degenerative phenotype. A transgenic system in which these topics can be studied would also be beneficial to the development of therapeutic approaches for retinal degeneration. In the past several years, the tetracycline-controlled transactivator (tTA)–mediated transcription-activation system has been shown to be one of the best controllable systems for in vivo studies. In fact, it makes use of an effective, specific, and nontoxic transactivator that can act in a variety of cell types[1] and transgenic animals.[2–4] In the absence of tetracycline, tTA activates transcription by binding to an array of tet operator sequences (tetO). In the presence of tetracycline, transcription is blocked, because tTA cannot bind to its target. This system, called tet-off, differs in two aspects from the tet-on system (rtTA) developed for the retina.[5] First, the tTA inactivates the transgene much faster, whereas the tet-on system quickly activates the transgene. Second, the dose–response of doxycycline (an analogue of tetracycline) on tTA shows an effective range at concentrations between 0.1 and 10 ng/mL, whereas on rtTA it is between 100 and 3000 ng/mL.[6] Furthermore, in the absence of antibiotic, the tet-off system regulates transgene expression according to the tissue-specific and developmental regulation of the promoter used. In addition, the transgene can be inactivated easily and at will with low concentrations of doxycycline. Finally, considering that an overexpression of wild-type VP16 fusion protein may not be well tolerated by the cells,[7] we used a VP16-derived minimal transactivator domain.[8] This 12-amino-acid VP16 minimal domain eliminates potential targets for interaction with other transcription factors, as well as potential epitopes that elicit a cell immune response. Furthermore, we can achieve twice the transactivator activity of the original tTA by using the modification tTA1.[8]

A cell-specific expression of tTA1 in rod photoreceptor cells can be obtained with the characterized rhodopsin promoter.[9] We generated transgenic mice in which a transgene can be specifically expressed in rod photoreceptor cells and can be easily inactivated at a defined time. In this article we present the characterization of two different transgenic mouse lines that express tTA1 in the photoreceptor cell layer, with temporal and spatial profiles similar to those of the endogenous rhodopsin gene.[9] We assessed the transactivator's activity and the response to doxycycline by mating the two transgenic lines with reporter mice (L7) carrying the luciferase gene downstream to tetO.[2] The results demonstrate that the two lines have different expression patterns of tTA1 in the photoreceptor layer. However, both lines responded quickly to doxycycline treatment. Therefore, our system can be used to express genes involved in retinal biology and pathology and is helpful in the study of pathogenetic events leading to retinal degeneration.

Material and Methods

Animals

The use of animals in this work was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Constructs

The 314-bp fragment from the *Eco*RI site to the ATG of the murine rhodopsin gene[9] was amplified from mouse genomic DNA using as

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primers oligo forward, 5′-GAGTTCAGGAGGAGACATTG-3′, and oligo reverse, 5′-GCTCTAGACATGGCTGCGGCTCTCGAG-3′. The PCR product was cloned in the pUHD21-1 vector upstream to the *tTA1* sequence, using the *Xba*I site.[8] We then cloned, at the *Eco*RI site, the 4.0-kb fragment (from *Kpn*I to *Eco*RI) of the rhodopsin promoter region (a kind gift from Muna Naash). This construct was named RhotTA1.

Production of the Transgenic Mice

Transgenic founder mice were generated by pronuclear injection, by standard techniques.[10] The transgene was excised as a 5.6-kb *Cla*I fragment, purified from agarose gel with kit (QIAquick Gel Extraction; Basel, Switzerland) and microinjected into pronuclei of one-cell embryos from superovulating CD-1 mice (Charles River, Sulzfeld, Germany). Transgenic animals were identified by PCR and Southern blot analysis of genomic DNA prepared from tail samples.[11] For PCR analysis *tTA*-specific primers were used: tTA forward (tTAf), 5′-ATGAGGTCGGAATCGAAGG-3′; and tTA reverse (tTAr), 5′-GGCATACTATCAGTAGTAGG-3′.

For Southern blot analysis, 12 μg genomic DNA was digested with the *Nsi*I restriction enzyme, cutting once within the transgene; fractionated on 0.8% agarose gel; and transferred onto a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech, Zurich, Switzerland). Blots were probed with the *tTA* PCR fragment labeled using a random prime labeling system (Rediprime II; Amersham Pharmacia Biotech).

Reverse Transcription—Polymerase Chain Reaction

RT-PCR was performed as previously described.[12] Total RNA was purified from retinas harvested from postnatal day (P)6, P7, P8, P9, and P13 and adult transgenic mice. tTAf and tTAr primers were used for PCR analysis.

Luciferase Assay

tTA1 transgenic mice (RhotTA1-L5 and RhotTA1-L32) were bred with luciferase reporter L7 mice .[2] Animals carrying both the transactivator (*tTA1*) and reporter (*L7*) genes were killed at different ages after birth. Eyes were enucleated and homogenized in 300 μL of 1× passive lysis buffer (Promega, Mannheim, Germany). A portion (20 μL) of the homogenate was used to assay luciferase activity (Luciferase Reporter Assay System; Promega) in a luminometer (AutoLumat; EG&G Berthold, Natick, MA). Luciferase activity was normalized to protein concentration and expressed as relative light units per microgram of total protein (RLU/μg). Transgenic mice were killed for assay at the following time points: P8 (RhotTA1-L5, $n = 6$; RhotTA1-L32, $n = 9$), P13 (RhotTA1-L5, $n = 6$; RhotTA1-L32, $n = 4$), and P20 (RhotTA1-L5, $n = 13$; and RhotTA1-L32, $n = 19$). Four other groups of P15 animals were exposed for either 1 or 10 days to doxycycline (Sigma, Milan, Italy) dissolved in their drinking water (2 mg/mL) containing 5% sucrose.

In Situ Hybridization

Mice eyes were harvested at P13 and P20, fixed in 4% paraformaldehyde, and embedded in paraffin. Sections (7 μm) were dewaxed and treated with 0.2% HCl for 15 minutes. Samples were incubated for 15 minutes in 20 μg/mL proteinase K, then washed with 0.4% glycine in PBS and postfixed with 4% paraformaldehyde. Acetylation with 0.2 M triethanolamine-HCl (pH 8.0) containing 0.25% acetic anhydride was performed twice for 5 minutes at RT. Slides were extensively washed in water and air dried. Sections were hybridized overnight at 65°C with 1 μg/mL digoxigenin-labeled riboprobes in 50% formamide, 1× Denhardt's solution, 3× SSC, 10% dextran sulfate, 500 μg/mL tRNA, and 500 μg/mL salmon sperm DNA.

The 450-bp *tTA* PCR fragment was used as a template for transcription, either with T3 RNA polymerase after linearization with *Xho*I (antisense probe) or with T7 RNA polymerase after digestion with *Xba*I (sense control probe). The *luciferase* antisense probe was obtained by digestion with *Pst*I and transcription with T3 RNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany), and the sense control probe was synthesized with T7 RNA polymerase (Roche) after digestion with *Xba*I.

After hybridization, slides were washed in 50% formamide and 2× SSC at 65°C and then equilibrated in NTE (0.5 M NaCl, 10 mM Tris-HCl [pH 8], and 5 mM EDTA), and treated with 20 μg/mL RNase A for 30 minutes at 37°C in NTE. After washing with 50% formamide, 1× SSC at 65°C and then with 2× SSC at RT, sections were blocked for 1 hour at RT with 1% blocking reagent (Roche) in MAB-T solution (100 mM maleic acid, 150 mM NaCl, 0.1% Tween-20 [pH 7.5]). Anti-digoxigenin-AP conjugate antibody (1:2000; Roche) in 1% blocking solution was incubated over night at 4°C. After extensive washes with TBS (100 mM Tris-HCl [pH 7.5], and 150 mM NaCl) and NTM (100 mM NaCl, 100 mM Tris-HCl [pH 9.5], and 50 mM $MgCl_2$), sections were exposed to the nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (NBT-BCIP) substrate for alkaline phosphate (Sigma). Reaction was observed with a microscope and blocked with 4% paraformaldehyde for 20 minutes. Slides were coverslipped with 70% glycerol in PBS and photographed using a microscope with Nomarski optics (Axioplan; Carl Zeiss, Oberkochen, Germany).

Histologic Analysis

Eyes enucleated from adult transgenic mice were fixed overnight in Carnoy's solution at 4°C, embedded using a kit (JB-4 Plus Embedding Kit; Polyscience, Inc., Eppelheim, Germany) and sectioned parallel to the sagittal plane. Sections (1 μm) were stained with 50% methylene blue and 50% azure II.

Electroretinograms

Animals were dark adapted for 12 hours and anesthetized with 100 mg/kg body weight ketamine and 5 mg xylazine. Pupils were dilated with 1 drop of a mixture of 1.7% tropicamide and 3.3% of phenylephrine. The ground electrode was a subcutaneous needle in the tail, the reference electrode was placed subcutaneously between the eyes, and

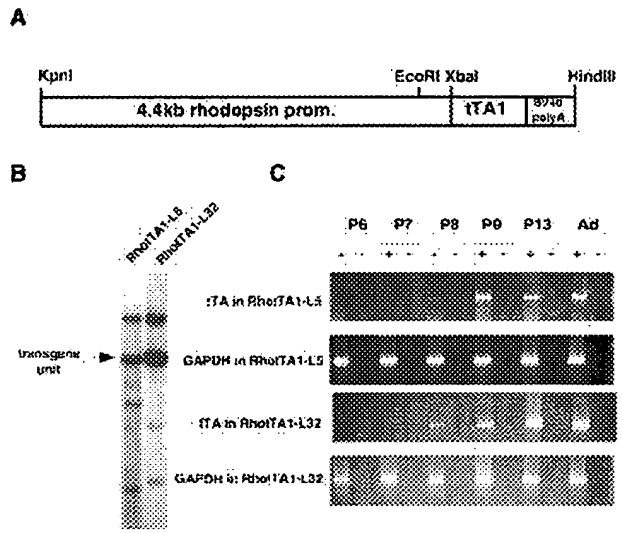

FIGURE 1. (A) Rho-tTA1 construct used to generate transgenic mice. The 4.4-kb genomic region of the rhodopsin gene, 5′ to the start codon, was cloned upstream to *tTA1* in the pUHD21-1 vector using *Kpn*I and *Xba*I restriction sites. The construct was engineered to replace the rhodopsin start codon with *tTA1* ATG. (B) Southern blot analysis of the two transgenic lines. Identical amounts of genomic DNA (12 μg) were loaded in the two lanes. *Arrow*: size of the transgene unit. (C) RT-PCR on cDNA from mouse eyes at different ages after birth (P6, P7, P8, P9, P13, and adult). (−) RT-PCR in the absence of reverse transcriptase.

the active electrodes were gold wires placed on the cornea below the pupil with a drop of methylhydroxypropyl cellulose (Methocel; Dow Chemical Co., Zürich, Switzerland). Measurements were performed on 6-month-old animals, with five mice in each group.

Recordings were made simultaneously in both eyes with a data acquisition system (Espion Console; Diagnosys LLC, Littleton, MA). The mouse was placed on a specially designed operating table that also contained the electrode mounts (High-Throughput Mouse-ERG; STZ for Biomedical Optics and Functiontesting, Tübingen, Germany) which could be introduced into a Ganzfeld LED stimulator (Espion Color-Burst; Diagnosys LLC). All electroretinographic (ERG) responses were obtained within 30 minutes after injection of anesthesia.[13]

Pulses of 10 ms were delivered at a frequency of 0.48 Hz. Results were obtained at 11 steps with illumination levels as indicated by the manufacturer's setting at 0.5×10^{-3}, 12.5×10^{-3}, 25×10^{-3}, 125×10^{-3}, 500×10^{-3}, 125, 5, 12.5, 50, 125, and 500 cd/sec per meter after 5 to 20 averaging.

A statistical analysis program (Matlab; the MathWorks, Natick, MA) was used to search for maxima and minima in predefined time windows to determine amplitudes, which were checked manually. Left and right eyes of all mice within one group were averaged for dark- and light-adapted ERGs separately. The Naka-Rushton fits for the amplitude of the b-wave[14-17] were iteratively performed by computer (Delphi software; Borland Software Corp., Scotts Valley, CA) to determine the values of n and k for each mouse, until a least-square fit over all luminance-levels reached a minimum. V_{max} is the maximum of the b-wave amplitude, k is the intensity at which the b-wave amplitude reaches half saturation, and n is a dimensionless constant related to the slope of the intensity-response function. The significance of differences between groups in all three parameters of Naka-Rushton fit and the a-wave amplitude were evaluated by Kruskal-Wallis followed by the Scheffe's F post hoc test.

Results

Generation of Rhodopsin Promoter tTA1 Transgenic Lines

We cloned 4.4 kb of the murine opsin regulatory region[9] upstream to *tTA1* substituting the ATG of rhodopsin with the *tTA1* start codon in the pUHD21-1 vector[8] (Fig. 1A). This construct was used to generate transgenic mice. Founders were screened by PCR and Southern blot analysis. One of the founders transmitted the transgene to the progeny. Comparison of the Southern blot patterns identified two different lines in which the transgene integrated in different genomic sites and in different copy numbers (Fig. 1B, arrow).

To investigate the temporal pattern of *tTA1* expression in the retina, we collected retinas from the two transgenic lines at different ages after birth. We performed RT-PCR analysis, using *tTA1*-specific primers. As shown in Figure 1C, expression of *tTA1* starts at 8 days after birth in both lines. Expression increases in the following days, and it is maintained until adulthood. It should be noted that expression in our transgenic

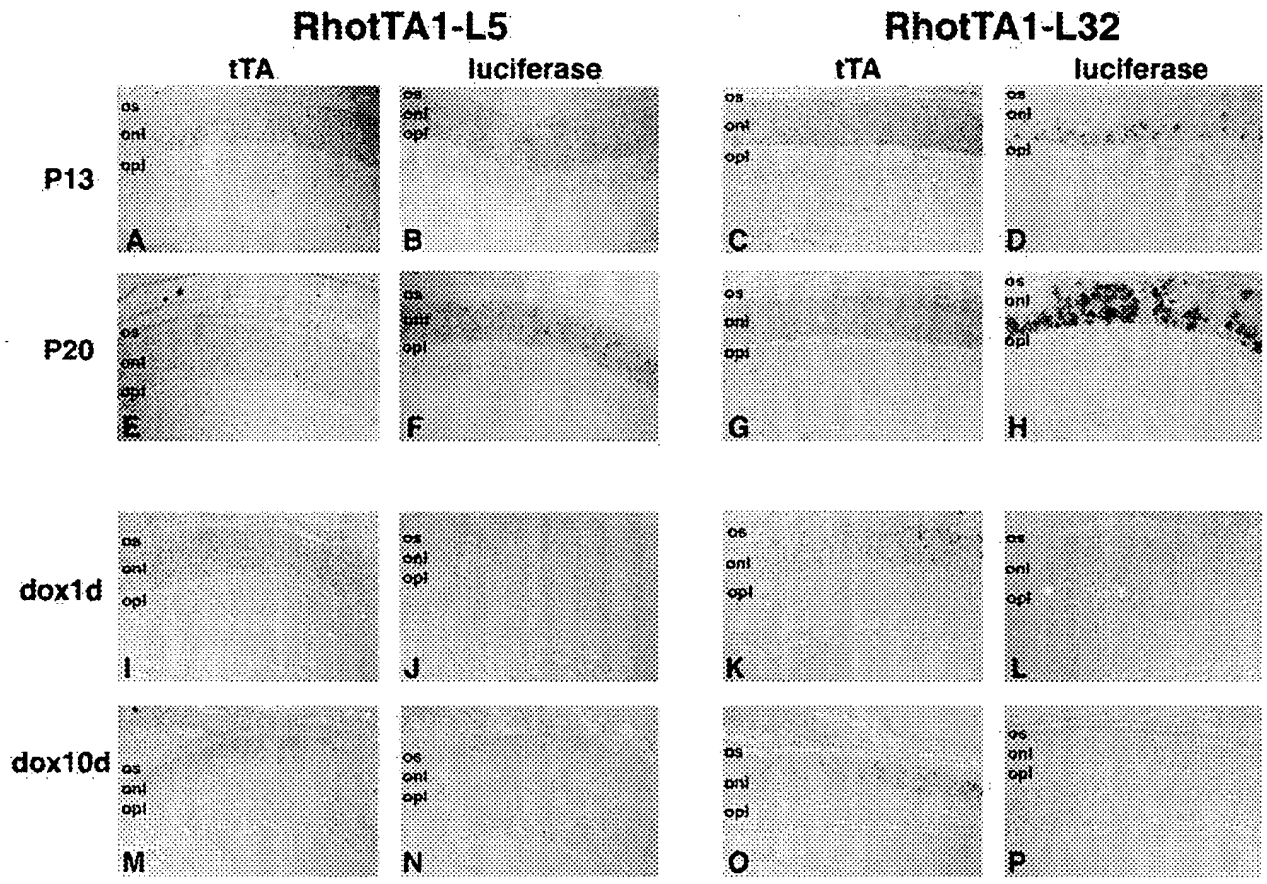

FIGURE 2. In situ hybridization analysis of double-transgenic retinas from RhotTA1-L5 and RhotTA1-L32 mice mated with L7 mice. Adjacent sections of P13 retinas (A–D) were hybridized either with the *tTA* probe (A, C) or the luciferase probe (B, D). Retinas from P20 mice (E–H) were analyzed, either with the *tTA* probe (E, G) or the luciferase probe (F, H). In all the samples, expression was restricted to the photoreceptor cell layer. P15 mice were fed with doxycycline (2 mg/mL). Luciferase expression was inhibited in both of the lines after 1 or 10 days of treatment with the antibiotic (J, L, N, P), whereas *tTA1* expression was maintained as expected (I, K, M, O). os, outer segment; onl, outer nuclear layer; opl, outer plexiform layer.

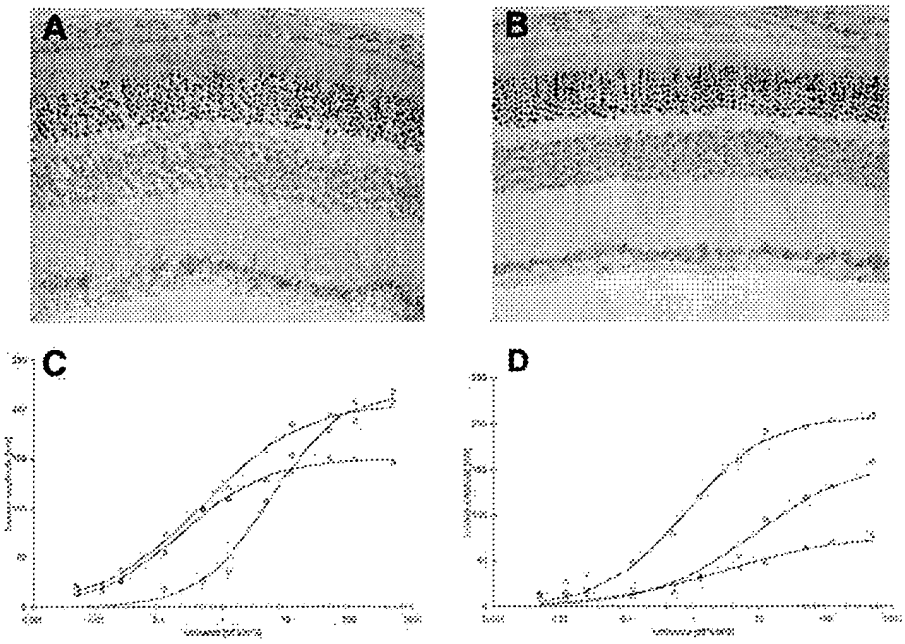

FIGURE 3. Histologic analysis of transgenic adult eyes. Seven-month-old RhotTA1-L5 (A) and 6.5-month-old RhotTA1-L32 (B) retinas were sectioned (1 μm) and stained with methylene blue and azure II. Dark-adapted (C) light-adapted (D) ERG parameter V_{max} measured on wild-type (□), RhotTA1-L5 (△), and RhotTA1-L32 (○) mice.

lines replicates the temporal expression pattern of the endogenous rhodopsin gene[18] and of other rhodopsin promoter transgenic lines.[9,19]

Specificity of *tTA1* Expression in Transgenic Mice

We analyzed expression of *tTA1* by in situ hybridization in sections from retinas harvested from the two transgenic mice. Expression of the transactivator was found only in the outer nuclear layer of the transgenic retinas (Figs. 2A, 2E, 2C, 2G). At 13 days after birth, *tTA1* transcripts were detectable in both lines (Figs. 2A, 2C), and, at P20, expression was evident and well distributed in the photoreceptor cells (Figs. 2E, 2G). When we analyzed contiguous sections from the temporal to the nasal side of the eye, we did not notice differences in distribution of the transcripts.

Expression of tTA1 did not interfere with retinal development and did not affect retinal morphology. In fact, histologic analysis of the retinal tissue in adult transgenic animals (6 to 7 months) confirmed that tTA1 did not cause morphologic anomalies in photoreceptor cells (Figs. 3A, 3B). Furthermore, we analyzed the retinas at the electrophysiological level. Table 1 shows a summary of the parameter estimates for the Naka-Rushton fits of the b-wave amplitudes in wild-type and transgenic mice. In the dark-adapted ERGs (Fig. 3C), analysis revealed no significant difference between wild-type and transgenic mice in any of the tested parameters for either the a- or b-wave. In the light-adapted ERG (Fig. 3D), the parameter V_{max} was not significantly different between the groups, except for RhotTA1-L5 compared with the wild type ($P = 0.0064$). No significant differences were observed between any of the groups in the parameter k and n of the Naka-Rushton fits. Finally, analysis of the a-wave amplitudes showed no statistical difference between the three groups (Table 2).

tTA1 Ability to Activate Transcription in the Retina

To investigate whether tTA1 activates in vivo transcription in the two transgenic lines, we mated the two lines with the L7 transgenic line bearing the luciferase reporter gene under control of tetO.[2] We then analyzed luciferase expression in the double-transgenic mice (RhotTA1/L7). The distribution of luciferase transcripts overlapped the *tTA1* mRNA expression pattern (Figs. 2B, 2D, 2F, H). As found with the *tTA* probe, expression was detected in the retinal outer layer, with some of the cells showing higher expression. This variability was more evident in RhotTA1-L32 (Figs. 2D, 2H). To quantify the transactivator activity in the two lines, we collected eyes from RhotTA1/L7 mice and assayed luciferase activity in protein extracts. As shown in Figure 4A luciferase activity in RhotTA1-L5 rose above background at P8 (tTA single transgenic mice show 0.24 RLU/μg) and quickly increased in the following days to reach a plateau at P13. In RhotTA1-L32, the kinetics of induction of luciferase expression appears to be slower, in fact at P8 luciferase activity is lower than in RhotTA1-L5. Expression increases at P13 and reaches its high-

TABLE 1. Summary of Parameter Estimates for b-Wave Analysis by the Naka-Rushton Fits

Genotype	Dark-Adapted ERG			Light-Adapted ERG			
	V_{max}	k	n	V_{max}		k	n
Wild type ($n = 5$)	219.10 ± 75.97	1.97 ± 2.22	0.69 ± 0.14	216.84 ± 49.03	**	5.97 ± 2.29	0.88 ± 0.26
RhotTA1-L5 ($n = 5$)	160.06 ± 87.20	0.45 ± 0.41	0.53 ± 0.17	80.94 ± 62.41		3.83 ± 1.50	0.49 ± 0.22
RhotTA1-L32 ($n = 5$)	219.51 ± 54.61	1.24 ± 1.43	0.59 ± 0.17	158.26 ± 49.24		9.15 ± 4.00	0.62 ± 0.10

Data are expressed as the mean ± standard deviation.
** : $P < 0.01$; all other comparisons were statistically non significant.

TABLE 2. Maximum a-Wave Amplitude in Each Group of Mice

Genotype	Dark-Adapted ERG	Light-Adapted ERG
Wild type (n = 5)	42.91 ± 22.26	28.61 ± 8.13
RhotTA1-L5 (n = 5)	54.10 ± 37.75	31.99 ± 11.96
RhotTA1-L32 (n = 5)	47.08 ± 16.34	41.41 ± 15.00

Data are expressed as the mean ± standard deviation in microvolts. All comparisons were statistically nonsignificant.

est level at P20 (Fig. 4B). Nevertheless, luciferase activity never reached the levels measured in RhotTA1-L5 mice.

We also performed luciferase activity assays in other tissues (tongue, liver, heart, lung, kidney, spleen, and brain) harvested from the double-transgenic lines. We did not measure significant activity in liver, kidney, and spleen. In tongue, brain, lung, and heart we detected low levels of luciferase activity, as previously reported for L7 single-transgenic mice.[2] The data suggest that expression of tTA1 is driven specifically in the outer layer of the retina.

Doxycycline's Effect on tTA1 Activity

The major advantage of the tTA1 system is that transcription can be blocked by the use of doxycycline. We therefore studied whether doxycycline treatment of RhotTA1/L7 mice blocks reporter gene expression. We added 2 mg/mL doxycycline in the drinking water of RhotTA1/L7 mice. We found that the response to the antibiotic was very rapid. In fact, 1 day of exposure to doxycycline was sufficient to block expression completely in RhotTA1-L5 mice (0.35 ± 0.07 RLU/μg ; Fig. 4A). Inactivation of tTA1 was maintained for a long time (10 days) by continued administration of the antibiotic (Fig. 4A). Luciferase activity after doxycycline treatment was comparable to the activity from L7 single-transgenic mice (0.5 RLU/μg). In RhotTA1-L32 mice, suppression was not complete after 24 hours (Fig. 4B). However, a longer exposure to doxycycline brought expression of luciferase to basal levels (0.66 ± 0.1 RLU/μg; Fig. 4B).

We also analyzed the effect of doxycycline at the histologic level, by using in situ hybridization. One day after the addition of doxycycline to the drinking water, luciferase mRNA was not detectable, whereas, as expected, expression of *tTA1* was not affected (Figs. 2I–P).

Finally, we studied whether we could induce tTA1 activity to resume. We withdrew the antibiotic from the drinking water after 3 days of treatment and tested luciferase activity at different time points. We found that, in 3-week-old mice, luciferase activity started to be detectable 4 days after we stopped the treatment (in RhotTA1-L5 mice it reached 51 ± 39 RLU/μg and in RhotTA1-L32 3628 ± 7730 RLU/μg). These data are in accordance with those of Robertson et al.[20]

DISCUSSION

We have generated an in vivo system to control gene expression in the retina. Classic transgenic approaches are not versatile enough, because of the persistence of transgene expression. To overcome this limitation, different inducible and repressible systems were developed to allow the control of transgene expression in a temporal and spatial manner. The use of such a system specific for the retina will be of great value in more clearly understanding the physiological changes occurring in retinal diseases and in developing therapeutic approaches. In fact, these studies will benefit from an in vivo model in which the effects of molecules can be tested by blocking their expression at will. For this purpose, we chose to express tTA1[8] in the retina. This transactivator is an improved version of the original tTA. It prevents the toxic effects of the VP16 transactivator domain and shows twice the transactivator activity. Furthermore, this system is very sensitive to doxycycline, even at low concentrations (0.1–10 ng/mL).[6] This is very important, because doxycycline is usually administered through the drinking water, and the local concentration in the tissues can be very low.

In this report, we present data on two different transgenic lines expressing tTA1 in rod photoreceptors. To assess the system we used three techniques: histology, in situ hybridization, and enzymatic assay. Histology of the retina from transgenic mice showed that expression of tTA1 did not cause morphologic changes in the photoreceptor cells. Furthermore, no statistically significant difference was found between wild-type and transgenic mice in the dark-adapted or, in other words, rod-dominated ERG, as indicated by the parameters V_{max}, k, and, n of the Naka-Rushton fits of the b-wave amplitudes. The a-wave analysis demonstrated that photoreceptor function was unaltered in the transgenic mice. A reduced V_{max}, observed solely in the light-adapted or cone-dominated state in RhotTA1-L5 mice, without any impairment of sensitivity (as shown by the parameters n and k of the Naka-Rushton fits) and without any reduction in a-wave amplitude could point to a limited alteration of the inner retinal layer or the synaptic transmission from the cone system to it. Considering the fact that rhodopsin is only expressed in rod photoreceptor cells, the difference in V_{max} can probably be attributed to the fact that CD1 mice are outbred or to the small sample size, which could not overcome interindividual variability. That the rod system of the transgenic mice was not altered in any of the electrophysiologically tested parameters is in good accordance with histologic findings.

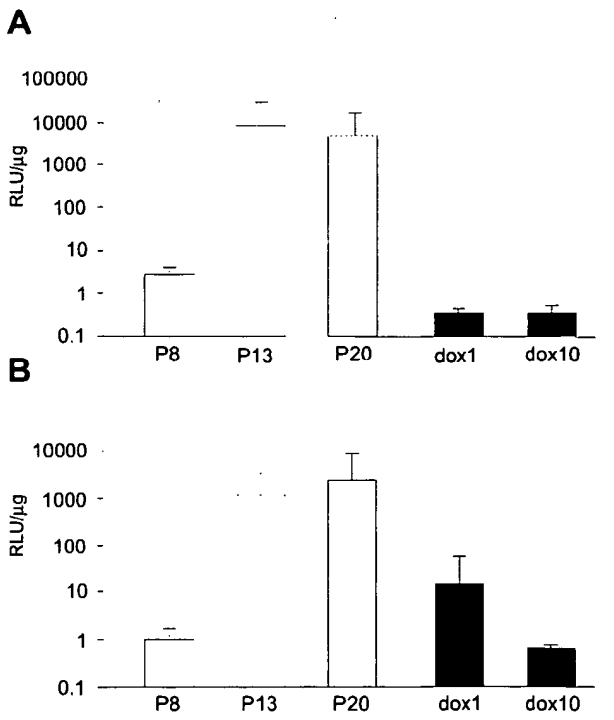

FIGURE 4. Luciferase enzymatic assays on double-transgenic RhotTA1/L7 mice derived either from RhotTA1-L5 (A) or RhotTA1-L32 (B) mice. Luciferase activity was measured at different ages after birth (P8, P13, and P20) and after exposure to doxycycline (2 mg/mL) for either 1 day (dox1) or 10 days (dox10). Luciferase background activity in the single-transgenic L7 line was approximately 0.5 RLU/μg total protein.

The cell specificity of transcription of the transgene was analyzed by in situ hybridization. Both lines showed specific expression of tTA1 in the photoreceptor nuclear layer. However, transcript distribution in the retina did not show a homogeneous pattern, and we detected photoreceptors highly expressing tTA1 among cells with lower level of expression. Heterogeneous expression of transgenes is a common phenomenon in mice and has been reported in many transgenic lines.[20–22] This effect was more evident in one of the two lines (RhotTA1-L32), whereas expression was more homogeneous in RhotTA1-L5. However, when we analyzed the distribution of expression in the entire retina we found that tTA1 activated transcription in the entire adult organ and transcription of tTA1 started at the same time as that of endogenous rhodopsin.[18]

Finally, tTA1 transcriptional activity was studied by breeding the transgenic lines with the L7 reporter line. We analyzed the ability of tTA1 to activate transcription in vivo in the retina by measuring the amount of luciferase activity. Based on an enzymatic luciferase assay, we defined good transactivation activity in both lines, demonstrating that the system works well in the retina. Then, by feeding the mice with doxycycline, we showed that a 1-day treatment is effective in completely inactivating the transgene in RhotTA1-L5. RhotTA1-L32 needs a longer exposure to the antibiotic to block the activity of tTA1. These data demonstrate that RhotTA1 transgenic mice are a versatile system to control expression in the photoreceptor cells. The rapid response to low doses of antibiotic makes these mice an effective tool for functional studies in retinal biology. Furthermore, this animal model represents an important component in the development of therapies that may be applied to human diseases in the future.

Acknowledgments

The authors thank Hermann Bujard for the pUHD21-1 vector, Ulrike Huffstadt and Caroline Johner for assistance in production of the transgenic mouse lines, Eberhart Zrenner for many discussions and constructive comments during our work, and Thorsten Schwarz for developing the programs for data evaluation.

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Inducible Podocyte-Specific Gene Expression in Transgenic Mice

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Abstract. The podocyte plays a key role in glomerular function and glomerular disease. To facilitate studies of podocyte function, we have developed a transgenic mouse model with inducible expression in the podocyte. The tetracycline-inducible transgenic system facilitates gene expression with restricted cellular distribution and tight temporal control. Recently, Bujard and colleagues have developed a functionally improved reverse tetracycline–controlled transcriptional activator (rtTA) with substantially lower background in the off state (the absence of tetracycline) and greater inducibility in the on state (the presence of tetracycline). We used the human podocin (*NPHS2*) gene promoter to control expression of the rtTA cassette and bred these mice with a reporter mouse line that contains the cytomegalovirus minimal promoter and *tetO* promoter elements together with *LacZ*, encoding β-galactosidase. Dual transgenic mice, bearing both podocin-rtTA and *tetO-LacZ* transgenes, had no detectable expression in kidney or other organs in the absence of tetracycline. Administration of tetracycline in the drinking water was associated with podocyte expression of β-galactosidase, in a fashion that was time dependent (maximal at 1 wk) and dose-dependent (maximal at 2 mg/ml). Podocyte expression was confirmed in two ways: histochemical staining for β-galactosidase and double-immunostaining using the podocyte marker WT-1 and β-galactosidase. This transgenic system should aid future investigations of podocyte function.

Glomerular visceral epithelial cells (podocytes) are highly differentiated post-mitotic cells whose function, notably their contribution to the glomerular filtration barrier, is largely based on their complex cytoarchitecture (1,2). Podocytes have interdigitating foot processes surrounding the glomerular capillary wall. They also possess a specialized intercellular junction between those foot processes, the slit diaphragm, which plays a major role in determining the permselectivity of the glomerular filtration barrier. Glomerular diseases associated with proteinuria typically are associated with podocyte injury, manifesting as loss of the slit diaphragm, foot process effacement, and loss of permselectivity (3). Other evidence of podocyte injury includes detachment from the glomerular basement membrane and apoptosis, leading to decreased podocyte number and ultimately focal segmental glomerulosclerosis (4,5). An alternative pathway of podocyte injury involves podocyte proliferation, dedifferentiation, and ultimately collapsing glomerulopathy (6). Further evidence of the importance of podocytes in glomerular disease is the recent discovery that various podocyte gene mutations are associated with glomerular disease. These include the genes encoding Wilms tumor gene (WT-1), nephrin, podocin, and α-actinin-4 (reviewed in (7)).

Studies of podocyte function *in vivo* would be aided by the ability to up-regulate or down-regulate gene function in a cell-specific manner. Recently, specific podocyte gene expression has been demonstrated using promoter elements derived from the mouse nephrin (*NPHS1*) gene and the human podocin (*NPHS2*) gene (8,9).

The tetracycline-controlled transcriptional activation system is a powerful tool in achieving temporal control of transgene expression in mammalian systems (10). These systems are based on regulatory elements derived from the *Escherichia coli* tetracycline-resistance operon. The original tetracycline-controlled transcriptional activator (tTA) is a chimeric protein composed of the Tet repressor protein and the VP16 transcriptional activation domain. The tTA activates transcription of the target gene in the absence of tetracycline, after binding to the Tet operator sequence (*tetO*) located in the 5' region of particular target gene (Tet-off system). On the other hand, the reverse tetracycline-controlled transcriptional activator (rtTA), composed of a mutant tetracycline repressor protein and VP16, binds to *tetO* and activates transcription in the presence of tetracycline (Tet-on system, Figure 1). Recently, new rtTA versions have been developed that are more stable in eukaryotic cells, function at a lower doxycycline concentration, and cause less background expression in the absence of tetracycline (11).

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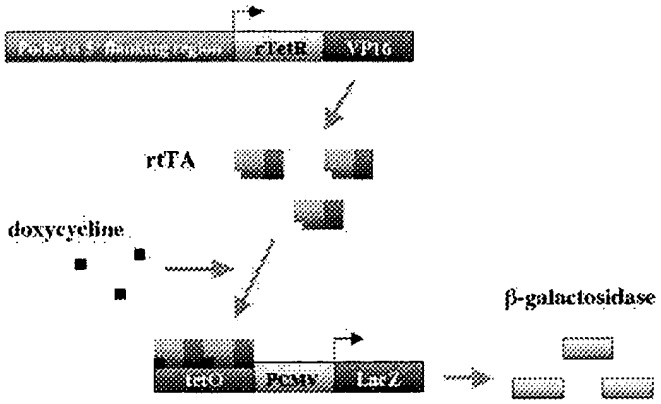

Figure 1. Reverse tetracycline-controlled transcriptional activator (rtTA) system (Tet-On system). A 2.5-kb fragment of the *NPHS2* promoter-enhancer region directs the expression of rtTA in podocytes. In the presence of tetracycline or derivative such as doxycycline, rtTA binds to tetracycline-response operon promoter element (*tetO*) and initiates transcription from the cytomegalovirus (CMV) promoter (PCMV) of the *LacZ* gene, encoding the β-galactosidase reporter gene in.

Tetracycline-controlled systems have been used to control transgene expression in mice (12–16), as reviewed recently (17). Inducible transgenic expression has several advantages over constitutive expression. First, transgene expression can be suppressed during embryogenesis to facilitate normal development, or can be activated during embryogenesis to probe developmental processes. Second, transgene expression can be set at different levels depending on tetracycline dose or can be activated for defined periods of time to allow for dose–response studies where the independent variable is either protein level or expression duration. Third, transgene expression can be coordinated with other interventions designed to induce or ameliorate renal disease. In the study presented here, we describe a tetracycline-controlled transgenic mouse model using the functionally improved rtTA under the control of the *NPHS2* promoter.

Materials and Methods

Generation of Transgenic Mice

The p2.5PodocinPE plasmid containing 2.5 kb of genomic sequence located 5' to the translation initiation codon of human *NPHS2* gene has been described previously (9). The construct, pUHrT62–1, containing the rtTA gene that is modified with humanized codon usage and the FFF minimal activator domains, was generously provided by Dr. Wolfgang Hillen, University of Erlangern, and Dr. Hermann Bujard, University of Heidelberg, Germany (11). The *NPHS2* promoter region was released from the p2.5PodocinPE plasmid by restriction digestion with *Xba*I and *Nco*I, and was blunt-ended by T4 DNA polymerase (Fermentas, Hanover, MD). The cytomegalovirus (CMV) minimal promoter was removed from the rtTA vector pUHrT62–1 by restriction digestion with *Xho*I and *Sac*II, and the remaining portion of pURrT62–1 was blunt-ended using T4 DNA polymerase. The *NPHS2* promoter was ligated into pURrT62–1 using the Quick Ligation Kit (New England Biolabs, Beverly, MA) to obtain the construct podocin-rtTA. The proper orientation of the clones was confirmed by restriction enzyme digestion mapping. The podocin-rtTA construct was released from the plasmid vector backbone by digestion with *Sca*I and *Hin*dIII, and purified by gel electrophoresis and DNA extraction using QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA). The purified construct DNA was introduced into the pronuclei of fertilized oocytes from the FVB/N mouse (National Cancer Institute, Bethesda, MD) by microinjection using standard techniques. All animal care conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and was approved by the National Institutes of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee.

Identification of Transgenic Mice

Transgenic mice were identified by Southern blotting using genomic DNA recovered from tail biopsies. Genomic DNA was isolated from the lysate of the tail of a 3-wk-old mouse using DNeasy Tissue Kit (Qiagen). Ten micrograms of the genomic DNA was digested with *Bam*HI and electrophoresed through a 1.0% agarose gel, transferred to a Nytran N membrane (Schleicher and Schuell, Keene, NH), and cross-linked with an ultraviolet Stratalinker (Stratagene, La Jolla, CA). The blots were prehybridized and hybridized in 15 ml of Hybrisol I (Intergen, Purchase, NY) at 52°C. The DNA blots were hybridized with ^{32}P-labeled cDNA probes for the human *NPHS2* promoter region.

PCR was also used for identification of transgenic mice, using the primer set Podoprobe-F: 5'CGCACTTCAGTTACTTCAGGTCCTC3' and Podoprobe-B: 5'GCTTATGCCTGATGTTGATGATGC3'. The thermocycling profile was as follows: initial denaturation 94°C for 30 s, then 30 cycles of 94°C denaturation for 30 s, 52°C annealing for 30 s, and 72°C extension for 30 s, and a final extension of 72°C for 5 min. Genomic template DNA was used in the range of 10 to 50 ng per reaction. The PCR product was detected on a standard 1% TAE-agarose gel as a 455-bp fragment. Podocin-rtTA transgenic founders were crossbred with *TetO-LacZ* mouse line (also termed RASSL mice, a generous gift of Dr. Bruce R. Conklin, University of California, San Francisco, California) (18).

β-Galactosidase Assays

Transgene expression was induced in mice by replacing normal drinking water with doxycycline-containing water supplemented with 5% sucrose to enhance palatability. Doxycycline (Sigma, St. Louis, MO) water was changed every 3 d. Quantitative assays for β-galactosidase activity in tissue homogenates were performed using a chemiluminescence assay as described by Moeller *et al.* (8) with a slight modification. Fresh tissues dissected from the mice were homogenized in lysis buffer containing 100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100, 1 mM dithiothreitol, and 1 tablet/50 ml of Complete EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN). Homogenization was performed for 20 s on ice using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA). After centrifugation at 12,500 *g* for 10 min, the supernatants were heated at 48°C for 50 min to inactivate endogenous mammalian β-galactosidase activity and centrifuged at 12,500 *g* for 5 min.

Fifty microliters of heat-inactivated homogenates were incubated for 60 min at 25°C with 300 μl of reaction buffer containing Galacton (Tropix, Bedford, MA), 100 mM sodium phosphate (pH 7.5), 1 mM $MgCl_2$, and 5% Emerald (Tropix). Light output was measured for 20 s using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) as an index of β-galactosidase activity. Protein concentration in the supernatants was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL) with BSA as a standard.

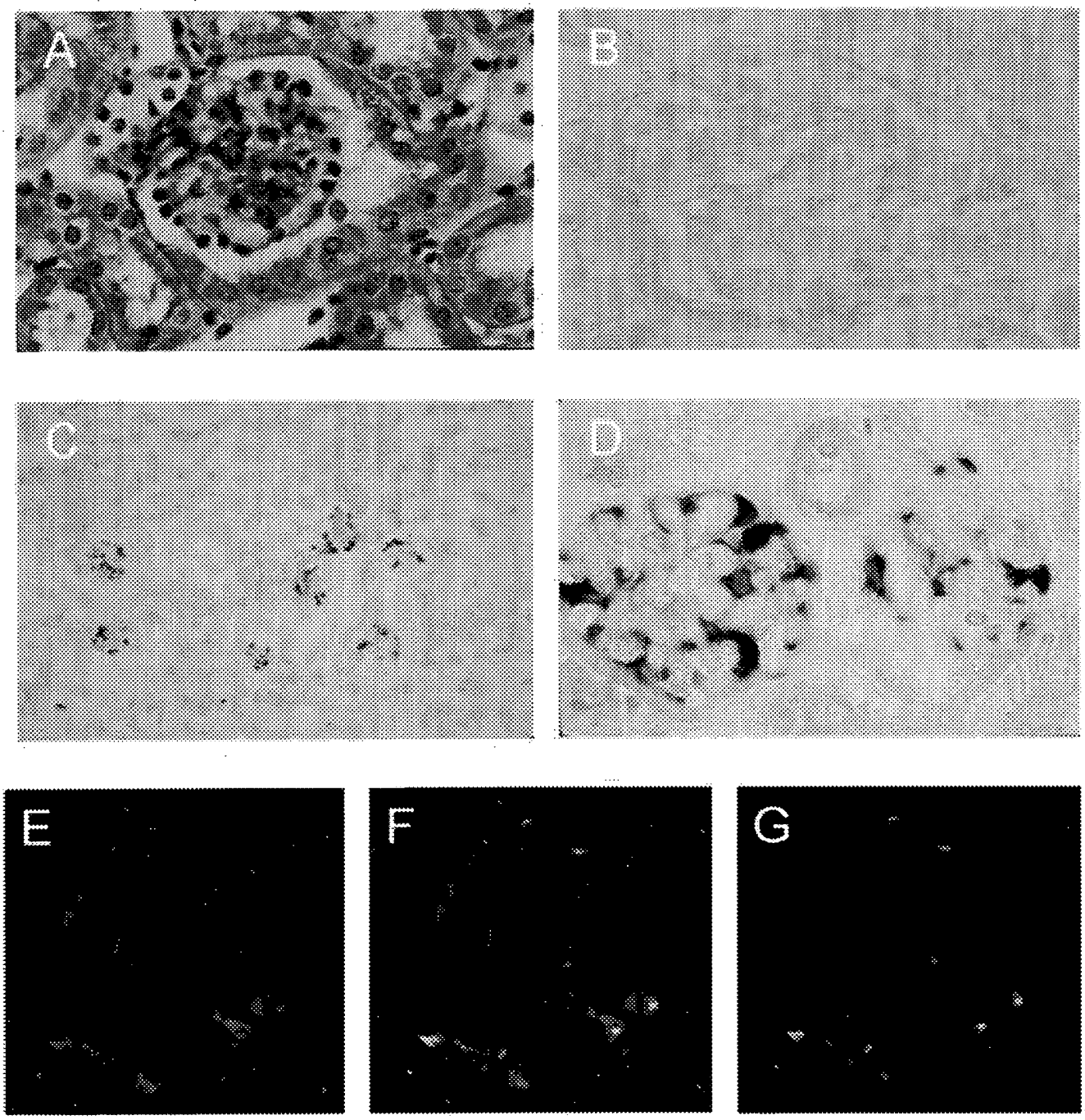

Figure 2. β-galactosidase expression in the kidney of podocin-rtTA X *tetO-LacZ* bitransgenic mice. (A) Normal appearance of kidney tissue from a bitransgenic mouse (PAS stain). (B) X-gal *in situ* staining from a bitransgenic mouse untreated with doxycycline. (C and D) β-galactosidase expression in doxycycline-treated bitransgenic mouse. Doxycycline was administered as a 2 mg/ml concentration in drinking water for 7 d. β-galactosidase was expressed predominantly in the periphery of glomeruli. (E, F, and G) Colocalization of β-galactosidase expression and WT-1. Cryosection of the kidney from the bitransgenic mouse treated with doxycycline was double-immunostained with Alexa 594-labeled anti-β-galactosidase antibody (predominantly cytoplasm, red) (E) and Alexa 488-labeled anti-WT-1 antibody (nuclei, green) (G). The two images were merged, demonstrating colocalization in podocytes (F). Magnification: ×200 (A), ×200 (B), ×40 (C), ×200 (D), ×300 (E to G).

For localization of β-galactosidase activity, tissues were fixed by intracardial perfusion of anesthetized mice using ice-cold 1.5% paraformaldehyde in PBS and 18% sucrose in PBS. The kidneys were resected, embedded in Histo Prep (Fisher, Fair Lawn, NJ), and frozen in isopentane chilled to −70°C. Cryosections were post-fixed with 4% paraformaldehyde in PBS (pH 7.8) for 5 min, and incubated overnight at 30°C in the staining solution containing 1 mg/ml 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal; Sigma), 5 mM

potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS (pH 7.8). The sections were counterstained with Nuclear Fast Red Staining Solution (Vector Laboratories, Burlingame, CA) for 1 min, mounted, and examined under bright-field microscopy.

Immunofluorescent Staining

Immunofluorescent staining was performed on the cryosections derived from the mice after perfusion with 1.5% paraformaldehyde in PBS and 18% sucrose in PBS. Sections were fixed with ice-cold acetone for 5 min, washed, and blocked with 10% donkey serum. Sections were incubated with goat anti-β-galactosidase polyclonal antibody (1:100 dilution; catalog number 4600–1409, Biogenesis, Kingston, NH) and rabbit anti-WT-1 polyclonal antibody (1:100 dilution; C-19 antibody, Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min. After washing, specimens were incubated with Alexa Fluor 594 conjugated donkey anti-goat IgG (1:100 dilution; Molecular Probes, Eugene, OR) and Alexa Fluor 488 conjugated goat anti-rabbit IgG (1:100 dilution; Molecular Probes). Fluorescence was examined using appropriate filters using a Leica DMR confocal microscope (Leica, Deerfield, IL) equipped with epifluorescent optics.

Statistical Analysis

Data are presented as mean ± SD.

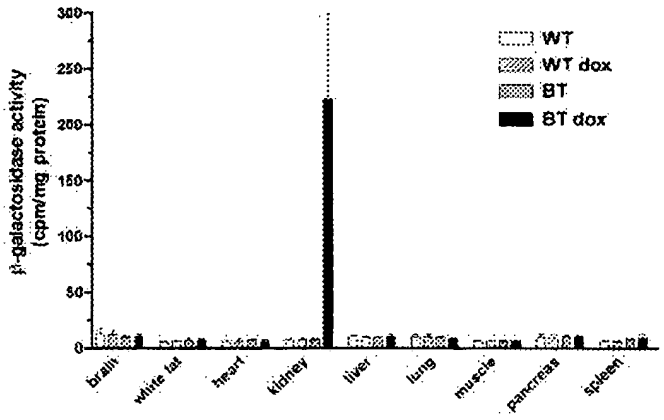

Figure 3. Kidney-specific transgene induction in podocin-rtTA X *tetO-LacZ* bitransgenic mice. Tissue homogenates from doxycycline-treated or untreated bitransgenic mice (BT) were tested for β-galactosidase activity by chemiluminescence and compared with values obtained from wild-type (WT) mice. Data are shown as means of counts per minute (cpm) normalized by the protein concentration of tissue homogenates derived from three animals. Transgene expression was induced with 2 mg/ml of doxycycline for 7 d.

Results

The Podocin-rtTA Transgene Expresses in a Kidney-Specific Fashion

Podocin-rtTA mice were generated using a transgene containing a 2.5-kb fragment of the human *NPHS2* 5' flanking region, the rtTA cassette, and SV40 3' untranslated region sequence including a polyadenylation sequence. Four founder mice were identified by Southern analysis. Two female founder mice were sterile. F1 offspring derived from mating each of two founding male podocin-rtTA line mice with wild-type FVB/N mice were crossed with heterozygous *tetO-LacZ* to obtain dual-heterozygous bitransgenic mice, which were identified by PCR. The structure and interaction of the podocin-rtTA and *tetO-LacZ* transgenes are presented in Figure 1.

Wild-type, podocin-rtTA, *tetO-LacZ*, and bitransgenic mice were provided with drinking water supplemented with doxycycline 2 mg/ml; control mice received plain drinking water. The gross and microscope appearance of renal tissue was normal in podocin-rtTA mice and in bitransgenic mice provided with doxycycline for up to 1 wk (Figure 2A). Tissue specificity of transgene expression was investigated by assaying β-galactosidase activity in tissue homogenates from selected organs. In mice derived from one founder line, high levels of β-galactosidase activity were detected in the kidney homogenates from doxycycline-induced bitransgenic mice, with no expression in other organs (Figure 3). In contrast, β-galactosidase activity was absent from all tissue homogenates from untreated bitransgenic mice and doxycycline-treated wild-type mice. Mice derived from the other founder podocin-rtTA mouse line did not express β-galactosidase, presumably due to transgene insertion into a transcriptionally silenced region of the genome, and these mice were not studied further.

Transgene Expression Is Detected Exclusively in Podocytes

To determine the spatial localization of *LacZ* expression in kidney, renal tissues were examined by X-gal staining of cryosections. β-galactosidase expression was detected in all glomeruli of specimens from podocin-rtTA X *tetO-LacZ* mice treated with doxycycline (Fig. 2C). Expression was most prominent in the periphery of glomeruli, in a localization most consistent with podocytes (Figure 2D). β-galactosidase activity was absent in bitransgenic mice not treated with doxycycline (Figure 2B) and mice bearing only one of the transgenes (data not shown).

Double immunofluorescence staining was performed in doxycycline-treated podocin-rtTA X *tetO-LacZ* mice, using Alexa Fluor 594-labeled anti-β-galactosidase antibody and Alexa Fluor 488-labeled anti-WT-1 antibody. Within the normal mature glomerulus, WT-1 is a specific podocyte marker, expressed predominantly in the nuclei (19). Superimposition of the two labels demonstrated that the cells expressing β-galactosidase (red), predominantly in the cytoplasm, also had nuclei expressing WT-1 (green) (Figure 2G). Essentially all podocytes expressed β-galactosidase.

Transgene Expression: Dose Response and Time Course

To investigate the ability to regulate transgene expression levels by the dosage of doxycycline, podocin-rtTA X *tetO-LacZ* bitransgenic mice received different doses of doxycycline via drinking water for 7 d. Induction of β-galactosidase activity in bitransgenic mice was detectable between 0.02 mg/ml and 5 mg/ml, and was maximal at 2 mg/ml (Figure 4A).

The time course of transgene expression level was examined after administration of doxycycline to bitransgenic mice. The

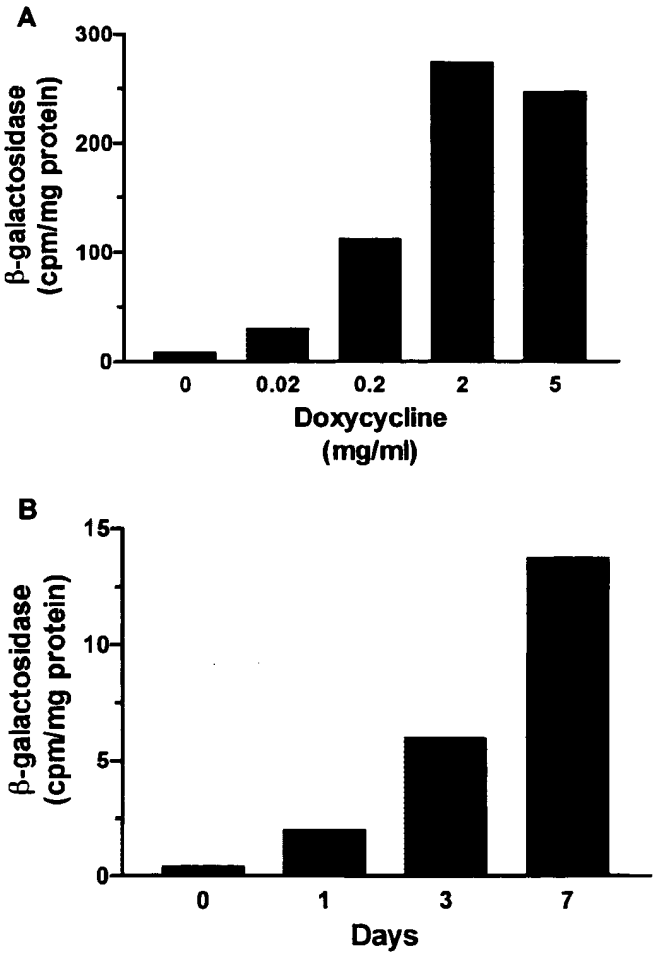

Figure 4. Dose-response and time-course of *LacZ* induction. (A) Dose-response curve of β-galactosidase activity of kidney tissue in podocin-rtTA X *tetO-LacZ* bitransgenic mice. Doxycycline was administered as different concentrations as indicated for 7 d before sacrifice. Kidney lysates were analyzed for β-galactosidase activity by chemiluminescence; the results shown are the mean of tissues derived from two mice. (B) Time-course of β-galactosidase activity of kidney from bitransgenic mice induced with 2 mg/ml of doxycycline for different durations. Background activity of kidney lysates from wild-type mice was subtracted from measured activity to yield specific activity. The results shown are the mean of tissues derived from two mice.

β-galactosidase activity of kidney homogenates from bitransgenic mice was detected even 1 d after doxycycline exposure, and continued to increase up to 7 d after exposure (Figure 4B).

Discussion

In this study, we report an inducible mouse model system that permits tightly-regulated and conditional expression of a reporter transgene in the podocytes of bitransgenic mice. Transgene expression was restricted to the kidney, among nine tissues examined, and expression in kidney was restricted to the podocyte. Essentially all podocytes expressed the transgene. Transgene expression was regulated by doxycycline in a dose-dependent and time-dependent manner.

There are multiple conceivable uses for an inducible transgene system that confers podocyte-specific expression. Transgene products might include cytokines or other peptides or anti-sense RNA molecule or siRNA molecules that might either exacerbate or ameliorate podocyte injury. Transgene expression can be turned on (and off) when desired, such as at a particular stage of embryonic development or in adult life. Transgene expression can also be manipulated in tandem with other experimental maneuvers to induce renal disease (such as partial nephrectomy, administration of nephrotoxic antiserum, or therapy to increase BP) or experimental maneuvers to ameliorate renal disease (such as administration of systemic medication or gene therapy). If the transgene product is toxic to the podocyte (such as by inducing apoptosis), this model might be used to induce podocytopenia. The system could be used to express *Cre* recombinase, to allow a tissue-specific knockout in an inducible manner. In all of these approaches, the amount of the transgene product can be varied by using different doses or different duration of doxycycline therapy.

In conclusion, this transgenic model of podocyte-specific transgene expression should prove useful in studies of podocyte biology, assisting efforts to elucidate the role of the podocyte in health and disease.

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TECHNOLOGY REPORT

Long-Term, Noninvasive Imaging of Regulated Gene Expression in Living Mice

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Summary: We describe here an approach for monitoring regulated gene expression by noninvasive imaging in living mice. We have utilized the tetracycline inducible system to simultaneously coregulate the expression of two genes encoding the firefly luciferase and the Cre recombinase, respectively. Results from our model system demonstrate that luciferase can be used as a noninvasive imaging marker for the regulated expression of a second gene in living mice. The integration of noninvasive imaging and inducible gene expression into current approaches of functional genomics should greatly advance our capabilities of carrying out highly controlled long-term studies of gene function in individual mice. genesis 29:116–122, 2001. © 2001 Wiley-Liss, Inc.

Key words: tetracycline; regulatable transcription; Cre recombinase; firefly luciferase; bioluminescence; transgenics

The mouse has become a widely accepted animal model in mammalian genetics, particularly after transgenesis and ES cell technology have drastically enhanced our possibilities to manipulate the mouse genome. Conditional genetic systems as recently developed add another level of sophistication (Sauer, 1998; Baron and Bujard, 2000). Actually, the potential to conditionally alter the activity of individual genes is beginning to provide fundamental insights into the cellular and molecular basis of complex biological processes such as development, diseases, and behaviour (Shin *et al.,* 1999; Yamamoto *et al.,* 2000; Tremblay *et al.,* 1998; Kelz *et al.,* 1999; Mayford and Kandel, 1999). In spite of the exciting perspectives opened up by systems that permit conditional activity of transgenes, concerns remain regarding the status of transgene expression, which may vary even among individuals of highly inbred mouse lines possibly due to epigenetic events. Moreover, in long-term studies the activity of a gene under outside control cannot necessarily be assumed as predictable, since transcriptional units may be subject to the dynamics of chromatin, which among others may cause transcriptional silencing. Together, such phenomena render interpretations of *in vivo* results difficult. Therefore, the possibility to monitor the expression status of a transgene noninvasively would greatly strengthen the reliability of interpretations in experiments with live animals. Recently, it has been shown that the firefly luciferase can be used as a noninvasive bioluminescent reporter in living mice using a photon imaging system (an Intensified Charged Coupled Device [ICCD] and image processor) (Contag *et al.,* 1997, 1998; Sweeney *et al.,* 1999). To exploit this technology for the above objective, we have combined the tetracycline controlled regulatory (Tet) system (Gossen and Bujard, 1992; Gossen *et al.,* 1995) with the *in vivo* luciferase monitoring principle. By placing the luciferase gene and a gene of interest under the control of P_{tetbi}-1, a bidirectional promoter (Baron *et al.,* 1995) that is responsive to tetracycline-controlled transactivators (tTA or rtTA; Fig. 1A), transgenic animals can be derived where luciferase activity serves as an indirect, noninvasive marker of the expression status of a target gene.

Here, we describe studies with the LC-1 mouse line where the luciferase gene *(luc)* and the gene encoding Cre recombinase *(cre)* are coregulated via P_{tetbi}-1. Individuals of this line (to be described in detail elsewhere) were mated with animals that produce tTA or rtTA under the control of three different promoters. While the human cytomegalovirus promoter IE, P_{hCMV}, gives rise to tTA activity in a variety of cell types (line TA^{CMV}-5) (Kistner *et al.,* 1996), the promoter of the liver-enriched activating protein P_{LAP} restricts tTA production to hepatocytes in mouse line TA^{LAP}-2 (Kistner *et al.,* 1996). In line rTA^{LAP}-1, the same promoter directs the synthesis of the novel $rtTA2^{S}$-S2 (Urlinger *et al.,* 2000) again primarily to hepatocytes, while in mouse line TA^{CaMK}-1 (line B in Mayford *et al.,* 1996) the promoter of Ca^{2+} α-calmoduline-dependent protein kinase controls tTA expression in the forebrain. Mice, double transgenic for the respective transactivator genes as well as for the *luc/cre* (LC) expression unit were studied, and Dox-dependent luciferase activity was monitored noninvasively upon intra-

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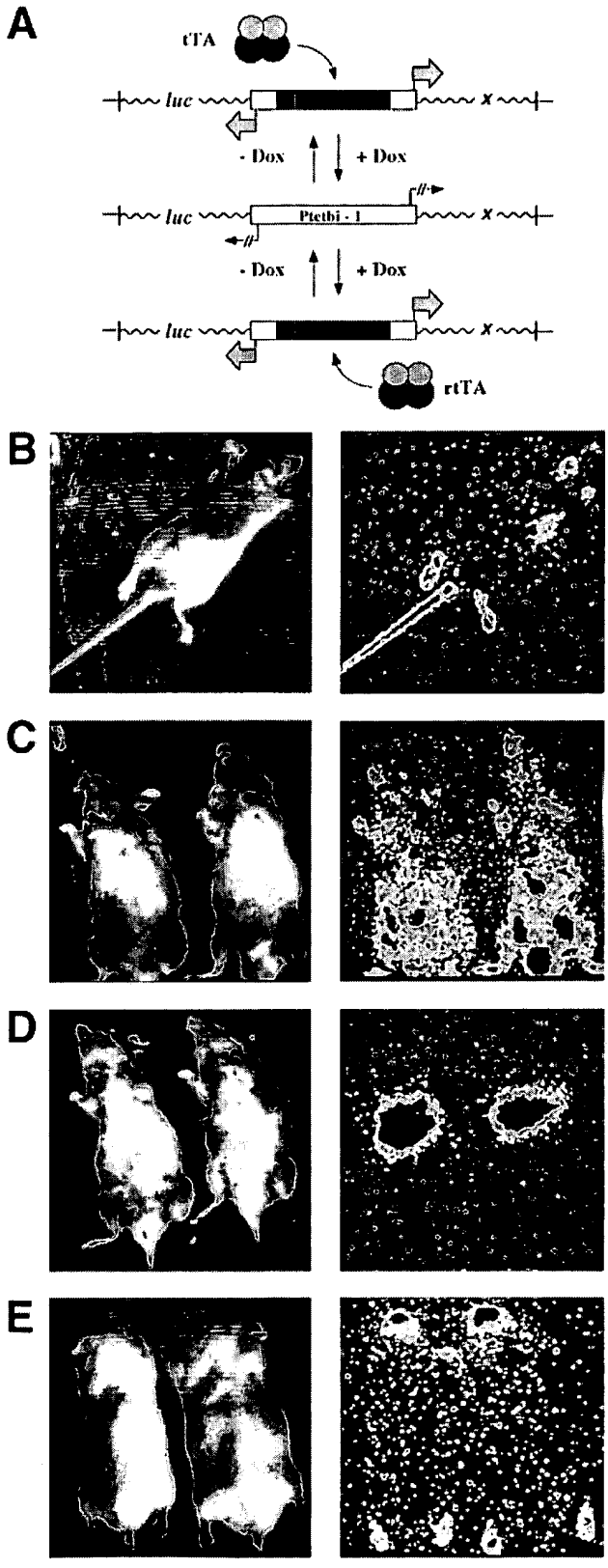

FIG. 1.

peritoneal (i.p.) injection of luciferin. Our data show that indeed the luciferase bioluminescence can be used as a reliable marker for the expression status of Tet-regulated genes in living mice.

As expected, TA^{CMV}-5/LC-1 mice showed luciferase activity in various inner organs and skeletal muscle (Kistner *et al.,* 1996) (Fig. 1B, C), whereas in TA^{LAP}-2/LC-1 and TA^{CaMK}-1/LC-1 animals bioluminescence is specifically restricted to liver (Kistner *et al.,* 1996; Lavon *et al.,* 2000) and brain (Mayford *et al.,* 1996), respectively (Fig. 1D, E). Interestingly, TA^{CaMK}-1/LC-1 mice also showed bioluminescence on footpads. No luciferase activity is detectable in plain LC-1 individuals (data not shown). When the sensitivity of the photon detection system was examined by partial activation of the luciferase gene in TA^{LAP}-2/LC-1 animals at different concentrations of doxycycline (Dox), bioluminescence caused by as little as 5-10 relative light units (rlu)/μg of protein in liver extracts was detectable in live mice (data not shown).

To study the time course of activation and inactivation of the luciferase gene via Dox in liver, we first determined the lowest concentration required to turn off luciferase activities to undetectable levels. TA^{LAP}-2/LC-1 mice were supplied with 2 and 20 μg/ml of Dox, respectively, in the drinking water for three days. Living mice and subsequently their liver explants were imaged, followed by in vitro determination of luciferase activities in liver extracts. Our results (Fig. 2A) show that 2 μg/ml of Dox in the drinking water were sufficient to reduce luciferase activity to background values resulting in a regulation factor of more than 10,000. In a longitudinal study, we performed multiple cycles of regulated gene activation by switching gene expression on and off. Luciferase activity was abolished within three days (2 μg/ml of Dox in drinking water) and fully restored seven days upon Dox withdrawal (Fig. 2B). These cycles of activation and inactivation of the luciferase gene were repeated with the same animals three months after the first study. The outcome (data not shown) was identical, indicating that the susceptibility of the bidirectional promoter to Tet regulation as well as expression of the tTA gene were maintained over long periods of time in these mouse lines.

Kinetics of gene activation in hepatocytes via Dox were examined with rTA^{LAP}-1/LC-1 animals where P_{LAP} directs the synthesis of $rtTA2^{S}$-S2. When these mice, which show no bioluminescence in absence of Dox, were given a single 2-mg dose of Dox by i.p. injection, gene expression was detectable after 1 h, reached maximum levels within 8 h, stayed on for another 16 h, and then turned back to background levels within 16 h (Fig. 2C). These results show the advantage of the rtTA principle where the supply of an efficiently penetrating small molecule like Dox quickly saturates an organ and activates gene expression. Moreover, the rapid depletion of Dox when given as a single dose makes short pulses of gene activation feasible, a feature that may be of interest for numerous physiological studies.

Making use of the TA^{CaMK}-1 mouse line, where P_{CaMK} restricts expression of tTA to the hippocampus, dentate gyrus, the striatum, and some layers of the cortex, we examined the cycle times of gene activation in the brain. Since 2 mg/ml of Dox in the drinking water are required to efficiently reduce luciferase activity in the brain (data not shown), animals were treated accordingly. After supplying the animals with Dox for 5 days, luciferase activity was undetectable by *in vivo* imaging but fully restored within 10 days upon withdrawal of the antibiotic (Fig. 2D). Analogous experiments were carried out with the rTA^{CaMK}-1 mouse line where P_{CaMK} directs the synthesis of $rtTA2^{S}$-S2 (Urlinger *et al.,* 2000). However, when double transgenic animals (rTA^{CaMK}-1/LC-1) were treated for five days with 2 mg/ml of Dox in the drinking water (Fig. 2E), luciferase activity was only detectable by imaging explanted brains or by assaying brain extracts as the concentration of Dox in the brain is reduced by the blood/brain barrier. Extended exposure to Dox for another 10 days had no effect, and the apparent regulation factor did not exceed 500. By contrast, when such mice were supplied with 10 mg/g of Dox in the food, high luciferase activity was monitored in live animals within five days (Fig. 2E). Comparing Dox treated to untreated mice revealed luciferase activity in brain tissue extracts that corresponds to a more than 10,000-fold regulation. We have also generated brain-specific mouse lines with the novel $rtTA2^{S}$-M2 (Urlinger *et al.,* 2000), and preliminary experiments indicate that full induction of gene expression can be achieved in the brain with 2 mg/ml of Dox in the drinking water of the animals (M. Hasan and H. Bujard, unpublished data).

To provide evidence that indeed *luc* and *cre* are coregulated within the LC transcription unit and that luciferase activities measured *in vivo* reflect faithfully the intensity of gene expression, we followed a time course of gene activation in animals where the two genes were expected to be simultaneously activated by $rtTA2^{S}$-S2 exclusively in hepatocytes (rTA^{LAP}-1/LC-1 mice). Dox (2

FIG. 1. *In vivo* imaging of luciferase gene activity controlled by P_{tetbi}-1, a bidirectional tTA/rtTA responsive promoter. **(A)** Tetracycline-controlled transactivators, tTA, or reverse tetracycline-controlled transactivators, rtTA, bind to an array of 7 *tet* operator sequences (red boxes) in absence (tTA) or presence (rtTA) of doxycycline (Dox) and activate two oppositely oriented RNA polymerase II minimal promoters (white boxes). P_{tetbi}-1 thus allows coregulation of the LC transcription unit consisting of the luciferase gene *(luc)* as a reporter and *cre* as the target gene *X*. Cell type-specific regulation is achieved by driving the expression of the transactivator genes with respective promoters. **(B, C)** Animals double transgenic for the tTA gene driven by P_{hCMV} and the LC construct raised in absence of Dox were imaged in different positions (exposure time 5 min). P_{hCMV} is active in numerous cell types (Kistner *et al.,* 1996). Geometric positioning of some animals influenced the external detection of internally generated photons, likely due to photon scattering by skeletal bones. **(D, E)** Mice double transgenic for the LC unit and for tTA driven by P_{LAP} or P_{CaMK}, respectively. The images show a clear restriction of luciferase expression to the liver (D, exposure time 1 min) and the brain (E, exposure time 5 min). Interestingly, the promoter of the αCaMKII conveys also luciferase activity to the foot pads.

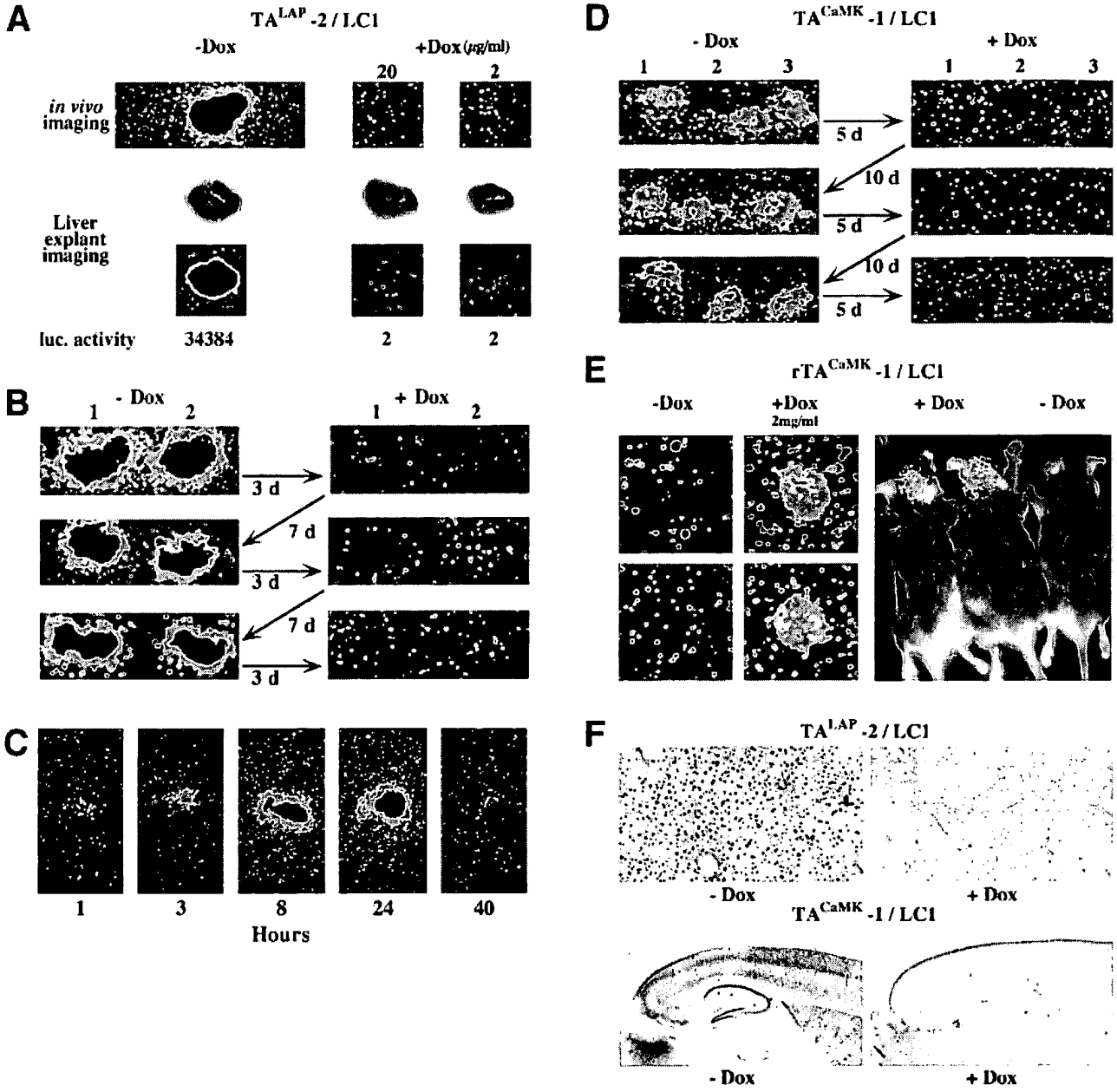

FIG. 2. *In vivo* imaging of Dox-regulated gene expression. **(A)** Luciferase activity in the liver of TA^{LAP}-1/LC-1 animals was scored in the presence and absence of Dox (2 and 20 μg/ml in the drinking water, respectively). Images were collected from whole animal and liver explants. The actual luciferase activity (in relative light units per μg of protein) was measured in liver extracts as described previously (Kistner *et al.*, 1996). **(B)** Cycle times (in days, d) of luciferase gene activation and deactivation via Dox was monitored *in vivo* in two animals. **(C)** Induction of luciferase by a single injection of 2 mg of Dox in rTA^{LAP}-1/LC-1 animals. Maximal luciferase activity is reached in approximately 8 h. **(D)** Cycle times of luciferase gene activation and deactivation in 3 TA^{CaMK}/LC-1 animals that were treated with Dox (2 mg/ml) in drinking water. **(E)** Imaging of luciferase activity in rTA^{CaMK}-1/LC-1 animals. Left, images of explanted brains from animals that received 2 mg/ml of Dox in drinking water. Right, *in vivo* imaging of animals treated with 10 mg/g of Dox in the food for 5 days; far right animal, untreated control. **(F)** Coexpression of CRE in liver (TA^{LAP}-2/LC-1) and brain (TA^{CaMK}-1/LC-1). CRE is visualized with polyclonal antibodies.

mg) was supplied by a single i.p. injection, and gene expression was measured at 0 time as well as after 1, 2.5, 4, and 8 h. As seen in Fig. 3, measurement of *in vivo* and *in vitro* luciferase activity correlated well with each other. Moreover, luciferase and CRE protein were both detected by Western blot as early as 4 h after induction. Recognizing the fact that different individual mice can be subject to experimental variation due to treatment

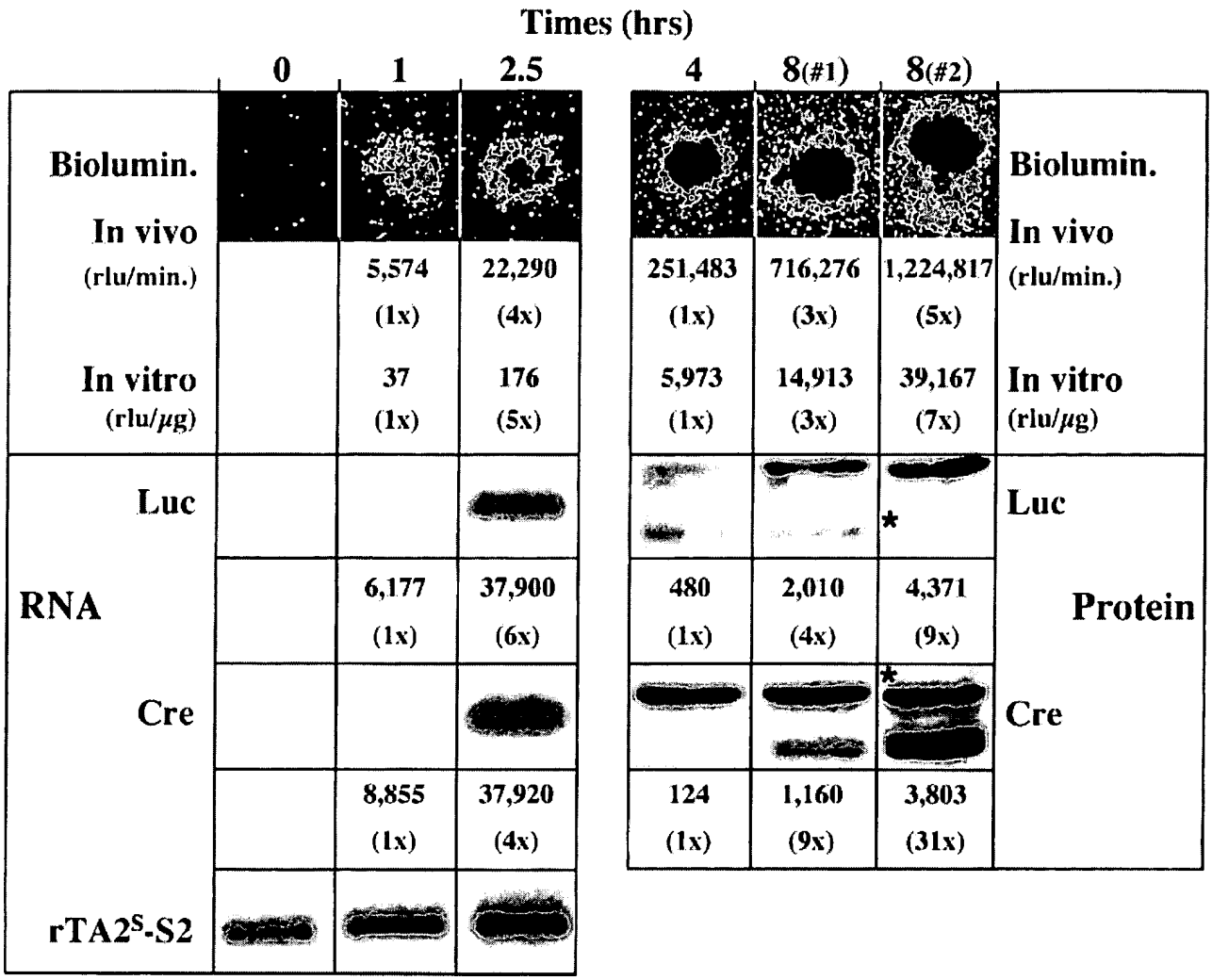

FIG. 3. Induced coexpression of *luc* and *cre* genes in the liver of rTA^{LAP}-1/LC-1 mice by a single i.p. injection of 2 mg Dox. Images of total photon intensity in living animals were integrated for a period of 1 min. *In vivo* and *in vitro* luciferase activities were quantified and presented as relative luminescent units/min (rlu/min) and relative light units per microgram total protein (rlu/μg), respectively. Luciferase activity was undetectable at 0 h. At 4 h, protein signals for the luciferase and Cre recombinase was detectable on the Western blot, and signal area was calculated as integrated pixel intensities after normalization with the constitutively expressed nonspecific signal (indicated by an asterisk). Relative fold changes from 4 h to 8 h are indicated in parentheses. Total RNA was prepared from livers of different mice at different time points, reverse transcribed, and quantified by SYBR-Green on an agarose gel. Areas for luciferase signals were calculated as integrated pixel intensities after normalization with the constitutively expressed rtTA2-S2 RNA signal. Quantified values at 1 h were taken as 1× and 2.5 h time points were calcuated relative to it as indicated in parentheses.

procedures and considering the technical challenge that goes in reliably quantitating changes in protein levels, we were pleased to find that the relative change in protein levels, between 4 h and 8 h (mouse #1 and #2), correlated well with *in vivo* and *in vitro* luciferase activities: an increase of 3- to 5-fold, 3- to 7-fold, and 4- to 9-fold for *in vivo*, *in vitro* luciferase activity, and protein levels, respectively (Fig. 3). During this period, the level of CRE protein increased by 9- to 31-fold, which is likely due to its longer half-life time. To possibly correlate the early appearance of luciferase activity with transcription of the two genes at time points where no protein is detectable yet, we determined the respective RNA levels by RT-PCR. Indeed, we could identify the two RNAs already at the 1-h time point. Their concentration had increased 4- to 6-fold, respectively, at the 2.5-h time point (Fig. 3). Relative fold changes of RNA levels correlated very well with *in vivo* (4-fold) and *in vitro* (5-fold) luciferase activities. These results confirm that (a) the *in vivo* and *in vitro* luciferase activities as well as RNA and protein levels are highly correlative and (b) the two genes, *luc* and *cre*, are co-expressed at an approx. 1:1 ratio in LC-1 mice. These results demonstrate furthermore that P_{tetbi}-1 is a very reliable promoter for reporting

the expression status of a target gene in long-term *in vivo* studies.

Our data show that, by coregulating the luciferase gene along with a gene of interest, the state of activity of a transgene can be monitored over long periods of time. As a first example, we show here the coregulation of the genes for luciferase and Cre recombinase in liver and brain (Fig. 2F). The enzymatic function of CRE in this experimental setup and its tight control will be described in a forthcoming publication.

This monitoring system opens up new possibilities for correlating phenotypes with gene activities. Since repeated administration of luciferin even within intervals of only 2 to 8 h had no apparent adverse effect on the animals and since luciferin has excellent tissue penetration properties—signals even in the brain are picked up in less than 5 min after injection—the rapid induction and short cycle times will greatly improve the precision by which active and inactive states of genes in vivo can be distinguished. Moreover, the sensitivity and the spatial resolution, the latter of which may be improved by new software that calculates the center of gravity of images, will allow one to distinguish activated genes in different compartments of the mouse body and even permit a first screening for tissue-specific expression in newly generated transgenic animals. Estimating the sensitivity of the detection system reveals that luciferase activity equivalent to 5 to 10 rlu/μg of protein can be detected in the liver of live animals. In HeLa cells, such an activity corresponds to around 50 luciferase molecules per cell (Kistner, 1996). We guess that the imaging efficiency in the brain is approx. 200-fold lower because the skull is a major barrier for external transmission of internally generated photons. We note that the short cycle times reported here, even for gene regulation in the brain, were measured with animals that were not raised under Dox. In the latter case, gene reactivation depends on the depletion of internal Dox pools, a rather slow process. The novel rtTAs of which $rtTA2^S$-M2 used here is just one representative will keep genes silent in absence of Dox while activation can be achieved at low effector concentration (Urlinger *et al.*, 2000). Thus, we anticipate that noninvasive imaging combined with Tet regulation will further the study of gene function *in vivo*.

MATERIALS AND METHODS

Mouse lines

TA^{CMV}-5, TA^{LAP}-2, and TA^{CaMK}-1 (line B) mouse lines were described previously (Kistner *et al.*, 1996; Mayford *et al.*, 1996). The synthetic gene encoding (Urlinger *et al.*, 2000) was placed behind P_{LAP} and P_{CaMK} as described for analogous constructs (Kistner *et al.*, 1996; Mayford *et al.*, 1996). The resulting mouse lines were designated rTA^{LAP}-1 and rTA^{CaMK}-1, respectively. Cre and luciferase genes were cloned into plasmid P_{tetbi}-1 to generate the LC transcription unit, which was used to generate the LC-1 mouse line (to be described in detail elsewhere). Transactivator mice were mated with LC-1 mice to generate double transgenic animals.

Imaging bioluminescence *in vivo*

Mice anesthesized with avertin were injected intraperitoneally with an aequous solution of D-luciferin (100 μg/g) and placed into a light-tight chamber. The emitted light was acquired by a photon counting camera (2-stage ICCD C2400-47) fitted with a Nikon lens (35 mm/f1.2) and a computer with image analysis capabilities (Contag *et al.*, 1997). Body images were recorded as reference in the daylight. Ten min after luciferin injection, the chamber was closed and the photon counting program was initiated for periods of 1–5 min. Photons were collected over time, generated images converted to pseudocolor, digitized with the Argus 20 image processor (Hamamatsu), and stored in a computer. The intensity of acquired images were calculated as relative luminescent units per minute (rlu/min).

Immunohistochemistry

Brain and liver were fixed for 2 h in ice-cold 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, and washed three times with cold PBS. One hundred- and 50-μm-thick slices (vibrotome) from liver and brain were processed to detect Cre recombinase as described previously (Kellendonk *et al.*, 1999).

RNA and Protein Measurement

Total RNA was prepared from livers according to instruction provided by the vendor (Qiagen). Equal amounts of total RNA (1 μg) were used to generate cDNA by RT-PCR (one step Qiagen Kit, 18 cycles) using a pair of primers for luciferase, cre, and rtTA2-S2 genes. RT-PCR products were analysed on a 2.5% agarose gel, and band intensities were quantified by SYBR-Green (Molecular Probes). Areas under each band were quantified using an image quantifying software after normalization with the constitutively expressed $rtTA2^S$-S2 RNA, and values were expressed as integrated pixel intensities (i.p.i). Liver lysate was prepared as described previously (Kistner *et al.*, 1996) and approx. 10 μm protein was separated on a 10% to 12% SDS-PAGE. After transfer, using semidry conditions PDVF membranes were incubated with primary rabbit polyclonal antibodies directed against luciferase and Cre proteins at a dilution of 1:2,000 and 1:10,000, respectively, followed by treatment with the secondary goat anti-rabbit antibody conjugated to alkaline phosphatase at a dilution of 1:10,000 (Sigma). Protein signals were detected by treating membranes to chemiluminescent substrate, CPD Star (Roche), and exposing it to Kodak X-ray films. Band intensity was calculated by image quantifying software and expressed in a manner as described above for RNA quantification except that the normalization was carried out using an internal nonspecific protein signal that was detected with rabbit polyclonal antibodies on the Western blot.

Doxycycline administration

Doxycycline (Sigma) was delivered to mice in the drinking water as described (Kistner *et al.*, 1996). Dox-containing food was prepared by dissolving 20-40 g of Dox in 500 ml of sterile water containing 40% sucrose. Five hundred grams of powdered mouse chow was added and mixed thoroughly to produce material containing 10-20 mg Dox per gram of food and 20% sucrose. Wet food was supplied and refreshed daily. Single-dose i.p. injections contained 2 mg of Dox in 0.5 ml of 0.1 M phosphate buffer, pH 7.5.

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Use of the Tetracycline System for Inducible Protein Synthesis in the Kidney

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Abstract. The great advantage of the tetracycline-inducible system lies in its ability to address a large variety of biological questions in a time-dependent and tissue-specific manner. This study describes a transgenic mouse line, rTA^{LAP}-1, which produces the reverse tetracycline transactivator under control of the liver activator protein (LAP) promoter. Two reporter lines with luciferase and *LacZ* reporter genes were used to demonstrate predominant expression in the kidney and liver when doxycycline was added to the drinking water. In the kidney, transgene expression was found primarily in cortical proximal tubules. No luciferase and β-galactosidase activity was detected in mice without doxycycline in the drinking water, which attests to the tight control of this system. One of the advantages of the tet system lies in its reversibility, and indeed, a virtually complete remission of transgene activity in both the kidney and liver was observed when doxycycline was withdrawn. Also examined was transactivator activity during development by exposing the mothers producing the reverse transactivator to doxycycline before mating. Transgene activity was detected in newborn kidneys and liver, indicating that sufficient amounts of doxycycline had crossed the placental barrier. During nephron development, the LAP promoter appeared to be only active in the more mature proximal tubules. Finally, the rTA^{LAP}-1 line was used to inducibly express the human PKD2 cDNA in proximal tubules of transgenic mice, but no cystic changes were detected, even after 6 mo of induction.

The understanding of gene function has been greatly aided by a multitude of transgenic mice. Conventional knockout mice, however, have the disadvantage that inactivation of the gene of interest may cause embryonic lethality or so severe a phenotype that it does not allow its analysis in all organs of adult mice. Therefore, a number of systems have been developed, which can either inactivate or overexpress genes conditionally in a tissue-specific and/or time-dependent manner. Of the few inducible expression systems that have been devised so far, the tetracycline-dependent system (tet system) probably has shown the greatest potential.

The tet system consists of two components. The first represents a tetracycline-dependent transactivator (tTA) driven by a specific promoter. The second contains tet operator sequences next to a minimal promoter, that control the transcription of the cDNA or gene of interest. Two versions of the tet-inducible system exist. In the original design, tTA binds to the tet operator only in the absence of tetracycline (1). In a subsequent version, the reverse tetracycline-inducible system (rtTA), a mutated tTA will bind to the tet operator only in the presence of tetracycline (2).

Although first established to study the function of genes in mammalian cell lines, the tet system soon also made a successful transition into animals (3,4). A similar extent of transgene expression has been achieved with both the tTA and rtTA systems; induction kinetics, however, were slower with the tTA system because withdrawal of doxycycline is necessary before transcription of the transgene can be induced. Recent modifications to the transactivator sequence have led to an improved tetracycline-dependent reverse transactivator (rtTA2^{S}-S2), which reaches the same activation levels as the original rtTA, while its regulatory range is tenfold enhanced as a result of its lower background activity (5).

A number of tet-inducible mouse lines have been generated (6), but none has been directed specifically to the kidney. For one line, in which the reverse transactivator is driven by the human cytomegalovirus promoter (rtTA^{CMV}-3), tet-inducible protein synthesis was described in many organs, among them the kidney (3). There, the transgene is specifically expressed in cortical and inner medullary collecting ducts (7). In this report, we describe another transgenic mouse line, in which an optimized reverse transactivator is only active in the liver and the kidney.

Materials and Methods

Transgenic Animals

Three strains of transgenic mice were used in our study. The reverse transactivator line rTA^{LAP}-1, in which the reverse transacti-

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vator is driven by the liver activator protein (LAP) promoter, has been previously described (8,9). It contains an expression cassette that consists of 2.8 kbp of the LAP promoter and a cDNA encoding the S2 version of the reverse transactivator (5). The first reporter line, LC1, contains a bidirectional expression cassette of the luciferase gene and Cre cDNAs under control of tet operator sequences (9). A second reporter line again contains a bidirectional expression cassette, but this time with the *nLacZ* gene (encoding β-galactosidase with a nuclear localization signal) and the human PKD2 cDNA under control of tet operator sequences. The latter was generated by cloning the human PKD2 cDNA into the *Pst*I and *Sal*I sites of the pBI3 plasmid (10). After the resulting construct was linearized with *Ase*I, it was isolated by gel electrophoresis, passed twice through an Elutip D column (Schleicher & Schuell, Dassel, Germany), and injected into fertilized oocytes of C57Bl/6 x DBA mice. All transgenic lines were maintained on a C57Bl/6 background. Founders were identified by screening tail biopsy specimens for the presence of *nLacZ* and PKD2 cDNA by PCR. The following oligonucleotide pairs were used: 5'-CAAACCATCGAAGTGACCAG-3' and 5'-CAATTTAACCGCCACTCAGG-3' to amplify a 388-bp fragment of the *nLacZ* gene; and 5'-ATTTGCAGATCTGTTCTCACATATCGG-3' and 5'-CTCTCAATCCTGGGGGAA-3' to amplify a 407-bp fragment of the PKD2 cDNA. The reaction conditions were 94°C for 4 min, followed by 25 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min. Thirteen out of 49 founders were positive for both *nLacZ* and PKD2. Of the 13 founders, only four were inducible for β-galactosidase activity.

Mice of either gender were used for our studies. For the induction kinetics (both for luciferase and β-galactosidase), mice were between 35 and 42 d old. Mice used in the withdrawal study were 6 mo old. For the dose-response experiments and the tissue survey, mice were 12 mo (no doxycycline) and 4 mo old (0.2 and 2 mg/ml of doxycycline).

Genotype Analysis

Genomic DNA was isolated from tail biopsy specimens of mice and subjected to Southern blot analysis according to standard protocols (11). To detect the presence of the *nLacZ* and luciferase transgenes, 10 μg of genomic DNA was digested with *Bam*HI and hybridized with the respective fragments; to detect the reverse transactivator transgene, genomic DNA was digested with *Eco*RI.

Doxycycline Administration

Doxycycline hydrochloride (Sigma, Deisenhofen, Germany) and sucrose (5% final concentration) were dissolved in water. The doxycycline-sucrose solution was prepared fresh every 3 to 4 d in a brown drinking bottle.

In Vivo *Bioluminescence Imaging*

Mice were anesthetized, injected intraperitoneally with 100 μg D-luciferin (Promega, Madison, WI) per gram of body weight, and immediately placed in a dark chamber. Luminescence was captured by a photon-counting camera (two-stage ICCD C2400-47; Hamamatsu Photonics Deutschland, Herrsching, Germany) fitted with a Nikon lens (35 mm/f1.2) for a period of 1 to 2 min. The images were then digitized with the Argus 20 image processor (Hamamatsu Photonics Deutschland) and later processed by Adobe Photoshop software (Adobe Systems, San Jose, CA).

Luciferase Assay

Tissues from adult mice were homogenized for 10 s in 500 μl of lysis buffer (20 mM DTT, 25 mM Tris pH 7.8, 2 mM EDTA, 10% glycerol, 1% Triton X-100) immediately after removal; in the case of newborn mice tissues were sonicated for 10 s in 100 μl of lysis buffer. After homogenization, the tissues were snap-frozen in liquid nitrogen and stored at −80°C. When luciferase activity was measured, the samples were thawed and centrifuged for 15 min at 14,000 rpm and 4°C. Ten microliters of the supernatant were combined with 250 μl of 25 mM glycylglycine, 15 mM $MgSO_4$, 5 mM ATP, 0.5 mM D-luciferin and assayed for 10 s in a Lumat LB9501 (Berthold, Wildbad, Germany). All measurements were performed in duplicate. An aliquot of the lysate was used to determine the protein concentration by means of an improved Bradford assay (12).

β-Galactosidase Staining

Adult mice were perfused through the distal abdominal aorta with 4% paraformaldehyde,1x PBS for 3 min at a pressure of 180 mmHg. The relevant organs were removed, sliced, and immersed in 18% sucrose,1x PBS until they sank to the bottom. Organs from newborn mice were removed without prior perfusion, cut into slices, fixed in 1.25% paraformaldehyde, 0.2% glutaraldehyde, and 1x PBS for 15 min and then immersed twice in 30% sucrose,1x PBS for 15 min each. All tissues were subsequently frozen in liquid nitrogen-cooled isopentane. β-Galactosidase staining was performed on 7-μm cryosections. The sections were equilibrated in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 20 mM NaCl, 10 mM EGTA pH 8.0, 10 mM sodium phosphate pH 8.0) and then incubated overnight at 30°C in the presence of 1 mg/ml of X-gal. The sections were washed twice in PBS and then either counterstained with eosin and mounted, or used further for immunohistochemistry.

Immunohistochemistry and Alkaline Phosphatase Staining

Immunoperoxidase staining was carried out according to the Vectastain ABC kit instructions (Vector Laboratories, Burlingham, CA). Primary antibodies were a rabbit polyclonal anti–Tamm-Horsfall glycoprotein antibody (Biotrend, Cologne, Germany; diluted 1:100) and a rabbit polyclonal anti-HA antibody (Sigma; diluted 1:2,000). β-Galactosidase staining was carried out overnight before immunohistochemistry. Alkaline phosphatase histochemistry was performed by exposing cryosections to the appropriate reaction solution (0.3 mM nitro blue tetrazolium chloride, 0.3 mM 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt in 0.2 M Tris·HCl, pH 9.5) for 25 min. The sections were subsequently washed twice with bidistilled water for 10 min and then mounted in bicarbonate-buffered glycerol pH 8.6. All sections were examined with a Leica Polyvar 2 microscope; pictures were taken with Nikon digital camera DXM1200. Finally, all images were processed with Adobe Photoshop software (Adobe Systems).

Cell Culture and Transfection Protocols

HtTA-1 cells, which are HeLa cells producing tTA, were stably transfected with the *Ase*I-linearized pBI3 plasmid containing the full-length PKD2 cDNA and the *nLacZ* gene by using a polyornithine protocol (13). Forty-eight hours after transfection, cells were plated onto 10-cm petri dishes and selected with puromycin (0.5 μg/ml; Calbiochem, Darmstadt, Germany). Approximately 2 wk later, resistant colonies were isolated and tested for β-galactosidase and polycystin-2 synthesis.

Protein Preparation and Western Blot Analysis

Proteins were prepared from cell lines by lysing the cells in a buffer containing 1% Triton X-100, 0.05% SDS, 150 mM NaCl, 10 mM Tris HCl pH 7.5, 2 mM EDTA pH 8.0, 1 μg/ml of leupeptin, 1 mM PMSF. After the protein concentration was determined, aliquots containing

30 μg of protein were analyzed by Western blot. To prepare protein from organs, 6-wk-old mice were placed on doxycycline for 2 wk; mice not receiving doxycycline were used as negative controls. Upon removal, kidneys and livers were added to 5 ml of a homogenization buffer containing 250 mM sucrose, 25 mM Tris pH 7.4, 5 mM EDTA, 2 μg/ml of leupeptin, 2 μg/ml of aprotinin, 1 mM benzamidine, 1 mM PMSF, 20 μg/ml TAME (*p*-tosyl-L-arginine methyl ester). After the tissues were homogenized with a motor-driven Teflon pestle, they were centrifuged for 15 min at 500 × *g* and 4°C to remove debris. An aliquot of the supernatant containing 50 μg of protein was analyzed by Western blot.

Proteins were run on polyacrylamide gels under denaturing and reducing conditions and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with the 12CA5 mouse monoclonal anti-HA epitope antibody (cell culture supernatant diluted 1:30). Final detection of the HA-epitope–tagged polycystin-2 protein was done with horseradish peroxidase–conjugated goat anti-mouse IgG Fab (diluted 1:10,000; Sigma) and the chemiluminescence reagents from NEN (Bad Homburg, Germany).

Immunocytochemistry and β-Galactosidase Staining of Cells

Cells were plated on glass coverslips and allowed to grow for an additional 3 d in the absence of doxycycline. After fixation with 2% PFA, 1x PBS, the cells were washed with 1x PBS and then stained for β-galactosidase activity in a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM $MgCl_2$, 1x PBS, and 1 mg/ml of X-gal. The next morning, the cells were washed with 1x PBS, permeabilized with 0.2% Triton X-100, 1x PBS, 2% BSA for 45 min and then incubated with the anti-HA–epitope antibody 12CA5 (diluted 1:30) for 2 h at room temperature. Subsequently, the cells were washed three times with 1x PBS and incubated for 60 min with Cy3-conjugated rat anti-mouse IgG antibody (Dianova, Germany; diluted 1:300). The coverslips were mounted and examined with a Leica Polyvar 2 microscope. Pictures were taken with Nikon digital camera DXM1200.

Results

In Vivo Imaging and Dose Dependency

The transactivator mouse line, rTA^{LAP}-1 (8,9), was created by using the optimized S2 version of the reverse transactivator (5) under control of the LAP promoter (14). Mice double-transgenic for $rtTA^{LAP}$-1 and the luciferase reporter gene were administered doxycycline at a concentration of 2 mg/ml in their drinking water. Using a noninvasive imaging system that can detect luminescence (8,15), it appeared that the LAP promoter was liver specific (Figure 1, a). However, after removing a number of organs and examining them for luciferase activity, pronounced luminescence also originated from the kidney (Figure 1, b), which could probably not be seen by noninvasive imaging because of the retroperitoneal location of the kidneys. The comparison of lysates from the kidneys with those from a number of other organs (*i.e.*, adrenal gland, brain, heart, intestine, lung, pancreas, skeletal muscle, spleen, testis, and thymus) showed at least 100-fold higher luciferase values in the kidneys (Table 1). A lower dose of 0.2 mg/ml of doxycycline in the drinking water resulted in a more than 90% reduction of luciferase activity in both the kidneys and the liver (Figure 1, c; note the logarithmic scale).

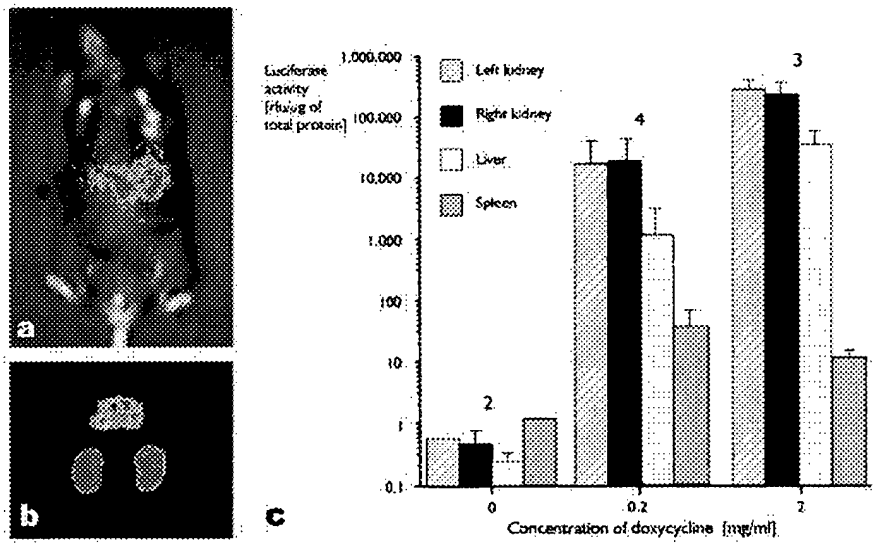

Figure 1. Imaging and dose dependency of luciferase activity. (a) Double-transgenic mice, which contained one expression cassette consisting of the reverse transactivator under control of the liver activator protein (LAP) promoter and a second expression cassette consisting of a luciferase cDNA under control of tetracycline-dependent system (tet) operator sequences, were administered doxycycline at a concentration of 2 mg/ml in the drinking water for 7 d. Then they were injected with D-luciferin and immediately placed into a dark chamber to collect photons. It can be seen by projecting the signal onto the mouse that most photons originated from the upper abdomen. (b) Upon removal of the liver and both kidneys, this luminescence could be attributed to the liver, but very strong luciferase activity was also present in the kidneys. (c) A dose-response experiment showed that administering doxycycline for 14 d at a concentration of only 0.2 mg/ml in the drinking water resulted in a pronounced decrease of luciferase activity. Data are presented as mean + SD; the number of mice is given above the bars.

Table 1. Luciferase activities in various tissues after 14 days of administration of doxycycline (2 mg/ml) in drinking water[a]

Site	No Doxycycline	Doxycycline
Adrenal gland	22.0	302.5
Brain	8.7	72.3
Heart	13.2	241.3
Intestine	16.7	812.9
Left kidney	5.4	255,382.0
Right kidney	5.5	207,783.1
Liver	10.4	32,075.5
Lung	15.3	91.7
Pancreas	17.4	1397.2
Skeletal muscle	36.4	38.2
Spleen	17.2	11.2
Testis	15.5	62.3
Thymus	25.1	78.5

[a] Values are given in relative light units/μg total protein (n = 2 mice without doxycycline, n = 3 mice with doxycycline).

Localization of the Tet Activator in the Kidney

To determine in which cells of the kidney the LAP promoter is active, mice double-transgenic for the reverse transactivator and the *nLacZ* gene were administered doxycycline at a concentration of 2 mg/ml in the drinking water for 14 d. Cryosections of the kidneys were first stained for β-galactosidase activity overnight, and then either subjected to alkaline phosphatase histochemistry or stained with antibodies against markers for the various nephron segments. β-Galactosidase activity was most prominent in the cortical portion of the proximal tubule (Figure 2, a), which became evident when costained for the brush border enzyme alkaline phosphatase, thus strongly suggesting that the LAP promoter was only active in the S1 and S2 but not in the S3 portion of the proximal tubule (Figure 2, b). It is noteworthy that quite often not all cells in the same tubular profile showed β-galactosidase activity; this mosaic pattern was seen in all animals examined. Only in approximately 20% of alkaline phosphatase–positive profiles all nuclei of a given profile produced β-galactosidase, whereas in approximately the same percentage of profiles no β-galactosidase-positive nuclei were detected (Figure 2, e). In addition to the cortex, there was some β-galactosidase activity in the inner stripe, suggesting transgene induction also in thick ascending limbs. Indeed, by costaining with an antibody against the Tamm-Horsfall glycoprotein, thick ascending limb profiles in the inner stripe and the cortex were identified that contained blue nuclei (Figure 2, c and d). We found no profile in the inner stripe in which all nuclei produced β-galactosidase, whereas in approximately 90% of the profiles, none of the nuclei was positive for β-galactosidase, which is in stark contrast to the situation in proximal tubules (Figure 2, e). Using antibodies against the thiazide-sensitive NaCl cotransporter (a marker of distal convoluted tubules) and against aquaporin-2 (a marker of collecting ducts), blue nuclei could very rarely be detected in cortical distal convoluted tubules and collecting ducts (data not shown).

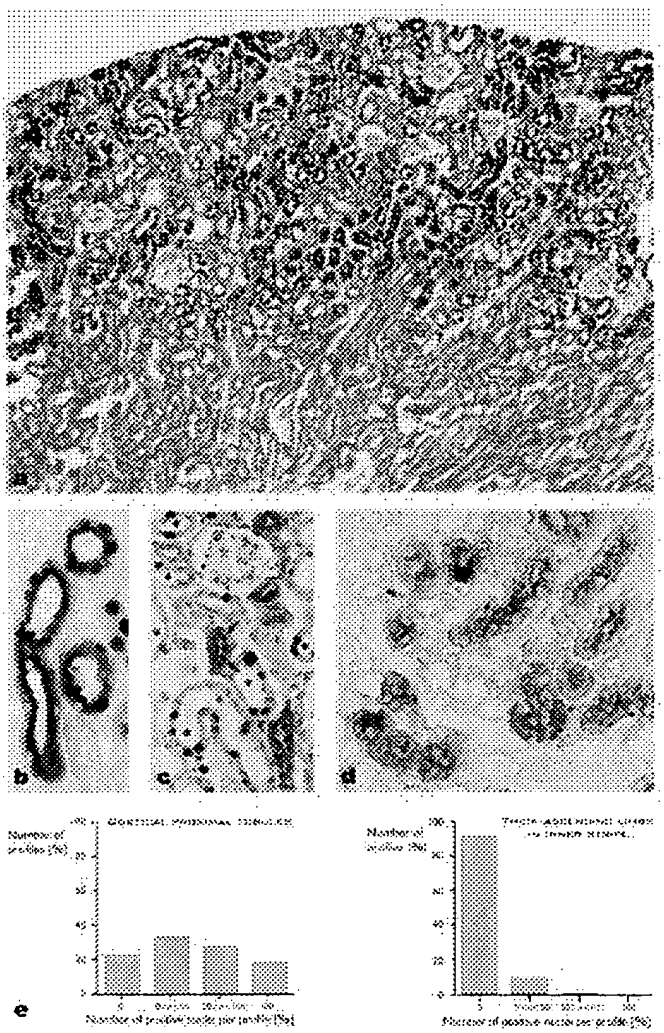

Figure 2. Localization of β-galactosidase activity in the kidney. Double-transgenic mice, which contained liver activator protein (LAP) promoter/rtTA2^S-S2 and tetracycline-dependent (tet) operator/*nLacZ* expression cassettes, were fed doxycycline at a concentration of 2 mg/ml in the drinking water for 14 d. (a) Histochemical staining for β-galactosidase activity and subsequent counterstaining with eosin demonstrates many blue nuclei in the cortex of the kidney. (b) Staining for β-galactosidase and alkaline phosphatase, a brush border enzyme, reveals that most β-galactosidase–positive nuclei are present in proximal tubules. Note that there are also some nuclei in proximal tubules, which did not turn blue. (c, d) When histochemical staining for β-galactosidase was combined with immunostaining against Tamm-Horsfall protein, a marker of thick ascending limbs, some blue nuclei were also detected in thick ascending limb cells in the cortex (arrows in c) and the inner stripe (d). Asterisks in c mark proximal tubules with many blue nuclei. (e) A careful quantitation substantiates that β-galactosidase–positive nuclei were much more frequent in proximal tubules than in thick ascending limbs, but it also demonstrates the considerable degree of mosaicism. Data are presented as mean values.

Induction Kinetics of Gene Expression

To test the induction kinetics of the rtTA^{LAP}-1 transgene in the kidney, female mice containing the LAP/rtTA2^{S}-S2 cassette were mated with males containing the luciferase and *nLacZ* reporter genes under the control of the tet operator. When the offspring were approximately 5 wk old, they were exposed to doxycycline for 1 to 14 d. Luciferase activity was then measured in the lysates from the right and left kidney, liver, and spleen of double- and single-transgenic mice (Figure 3).

As expected, instrument background was detected for the transgenic animals containing only the reverse transactivator. The tightness of the system became evident by the fact that the organs containing only the luciferase gene, and the organs from double-transgenic animals with no exposure to doxycycline also only showed background activity. After 1 d of doxycycline administration, however, a high luciferase activity of remarkably similar levels was seen in the right and left kidney of double-transgenic mice, thus demonstrating a uniform induction and validating our approach. As expected, luciferase values rose with increasing exposure to doxycycline (Figure 3, a). The same was seen for the liver, where induction was at

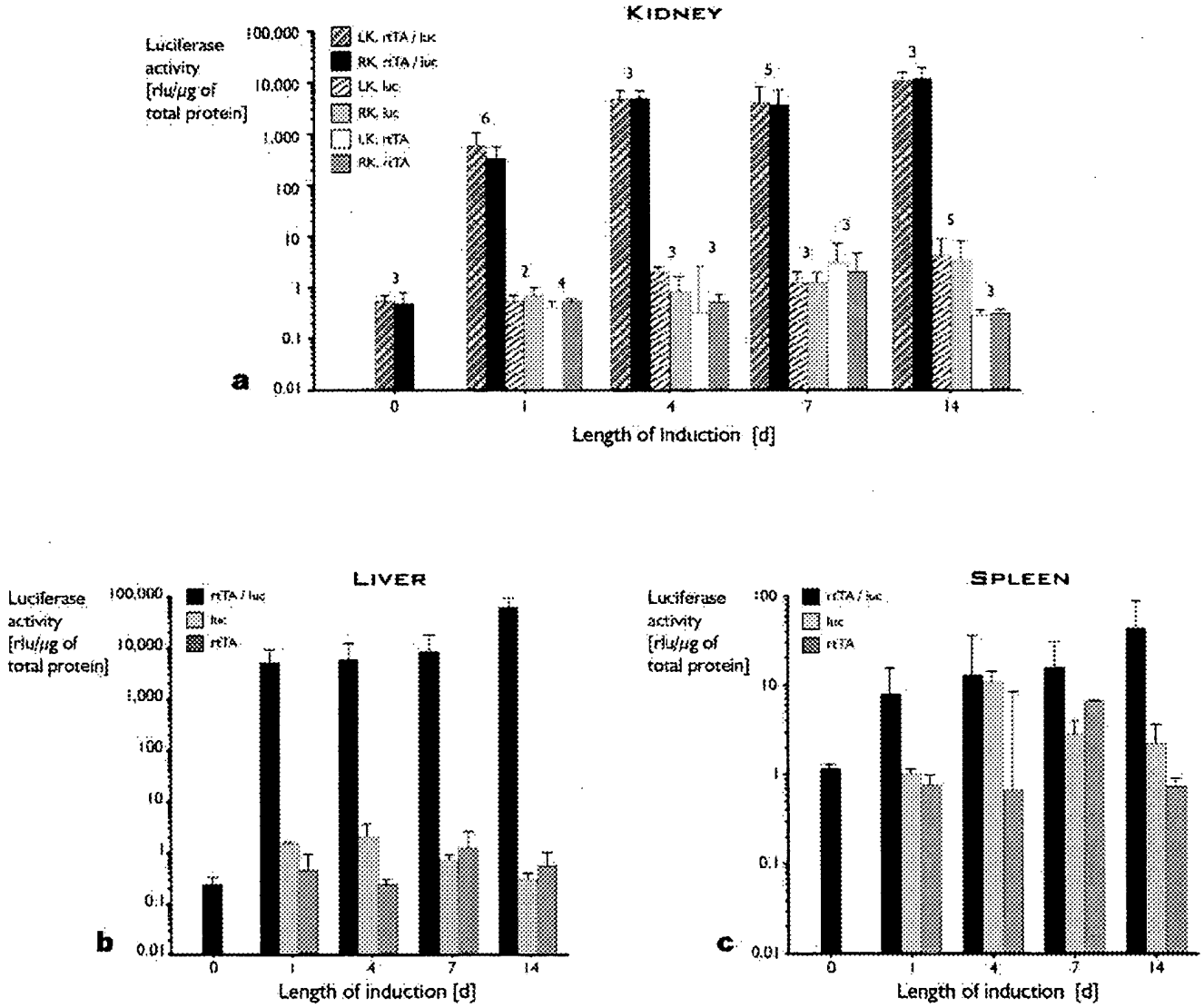

Figure 3. Kinetics of luciferase induction in adult mice. Mice containing only the liver activator protein (LAP) promoter/rtTA2^{S}-S2 expression cassette (rtTA), only the tetracycline-dependent (tet) operator/luciferase expression cassette (luc), or both expression cassettes (rtTA/luc) were fed doxycycline in the drinking water for 1, 4, 7, and 14 d before both kidneys, the liver, and spleen were removed. Already after 24 h on doxycycline, pronounced luciferase activities were observed in the kidneys (a) and the liver (b), whereas only a very modest luciferase activity was measured in the spleen, even after 14 d of doxycycline administration (c). The tightness of the system is evident from the very low luciferase activity of rtTA/luc mice without exposure to doxycycline and from equally low luciferase activity of luc mice exposed to doxycycline for 14 d. RK and LK, right and left kidney, respectively. Data are presented as mean + SD; the number of mice is given above the bars.

least 10,000-fold higher than background after 14 d of doxycycline administration (Figure 3, b). The spleen, which was used as a control tissue, showed less than 1% of the hepatic and renal luciferase values (Figure 3, c).

The same time course was used to investigate the induction and location of β-galactosidase activity. β-Galactosidase positive nuclei were already seen after 1 d of doxycycline administration, although we could not detect more than five blue nuclei per section in the cortex of the kidney. The longer the exposure to doxycycline, the more cells became induced, which correlated well with the luciferase data. Apparently tubular cells in the cortex reacted first, whereas β-galactosidase positive cells were fewer in the inner stripe and took longer to be induced (data not shown).

Developmental Kinetics of Gene Expression

Because we were able to control gene expression with doxycycline in adult kidneys, we also wanted to see if this was possible during development. For this part of the study, the mothers carrying the LAP/rtTA2^{S}-S2 transgene were placed on doxycycline 1 wk before mating. The offspring were analyzed on the day of birth as well as 7, 14, and 35 d after birth. A high level of luciferase activity was detected in newborn mice, indicating that sufficient amounts of doxycycline had crossed the placental barrier. Luciferase activity in the kidneys of newborn mice was similar to that in the kidneys of adult mice after 1 d of exposure to doxycycline. However, 1 and 2 wk postpartum, luciferase activity was markedly reduced, suggesting that doxycycline was not transduced at sufficiently high concentrations through the mothers' milk (Figure 4, a). In livers of newborn mice, luciferase activity was equivalent to that of livers from adult mice after being induced for 14 d. A pronounced decline in activity was also seen in the liver over the first couple of weeks, similar to that in the kidney (Figure 4, b). However, in both hepatic and renal tissues, a subsequent increase can be seen in 35-d-old mice similar to the levels seen in tissues from adult mice after 14 d of doxycycline administration (Figure 4, a and b). A similar pattern, although at much lower levels, was also observed in the spleen (Figure 4, c). β-Galactosidase histochemistry revealed that the induced cells in newborn kidney were limited to the more mature tubular profiles because no blue nuclei could be detected in the nephrogenic zone below the capsule (Figure 4, d). No blue nuclei could be detected in the kidneys of 14-d-old mice (data not shown).

Persistence of Transgene Expression

One of the attractive features of the tet system lies in its reversibility, which depends on the decrease of doxycycline levels after removal as well as on the half-life of the mRNA and protein encoded by the transgene. We therefore analyzed the decline in luciferase levels of double-transgenic mice, which were treated with doxycycline for 14 d. When doxycycline was removed from the drinking water, luciferase activity in the liver decreased to background levels within 14 d after discontinuation of doxycycline (Figure 5). Luciferase activity in the kidneys had fallen 100-fold after 14 d and to just above background levels after 35 d. Again, β-galactosidase activity mimicked the results of the luciferase assays because blue nuclei were still present in the kidneys 14 d after removal of doxycycline; no blue nuclei were detected in the kidney after 35 d of withdrawal (data not shown).

Inducible Expression of PKD2

To test the inducibility of the bidirectional expression cassette containing the human PKD2 cDNA and the *nLacZ* gene in the tet system, stably transfected HeLa cell lines were generated that inducibly produce full-length HA-tagged polycystin-2 and β-galactosidase in the absence of doxycycline. The clones were tested first for β-galactosidase activity and then for polycystin-2 immunoreactivity. All β-galactosidase-positive cells also stained positive for HA-tagged polycystin-2 protein (Figure 6, a and b). These immunocytochemical findings were subsequently confirmed by Western blot analysis. It can be clearly seen that the PKD2 cDNA is switched off in the presence of doxycycline and induced upon withdrawal of doxycycline (Figure 6, c).

Mice double-transgenic for the reverse transactivator and the tet operator/PKD2/*nLacZ* expression cassette were administered doxycycline in the drinking water for 14 d, animals without doxycycline in the drinking water served as a negative control. When protein lysates from the kidney and liver were subjected to Western blot analysis, the epitope-tagged polycystin-2 protein was only detected in animals receiving doxycycline (Figure 7, a). Analysis of cryosections revealed that the expression of PKD2 was most pronounced in the proximal tubule, therefore mirroring the β-galactosidase staining (Figure 7, b and c). To investigate whether the overexpression of the human PKD2 cDNA would lead to alterations in the kidneys, we administered doxycycline long-term starting at 8 wk after birth. Even after 6 mo, however, no morphologic changes were visible (Figure 7, d and e).

Discussion

The aim of our study was to characterize the transgenic mouse line rTA^{LAP}-1, which allows the inducible and reversible expression of transgenes predominantly in proximal tubules. In this line the tetracycline-inducible transactivator is driven by the LAP promoter. This promoter based on the analysis of other transgenic lines, was previously thought to be liver-specific (3). It was therefore somewhat unexpected to detect transactivator activity in the kidney, which, however, was a stable effect because we observed renal expression in many crosses and over six generations. Whether this particular expression pattern of the rtTA2^{S}-S2 transgene is due to a positional effect of its integration site remains a matter of speculation.

Strongest transgene expression was detected in the cortical proximal tubules, although transactivator activity in other parts of the nephron was also observed. Expression in thick ascending limb cells was variable and seemed to depend on the length of induction and on the tet operator line used, while there was consistently strong expression in proximal tubules independent of the tet operator mouse lines. Such a pattern of tet operator

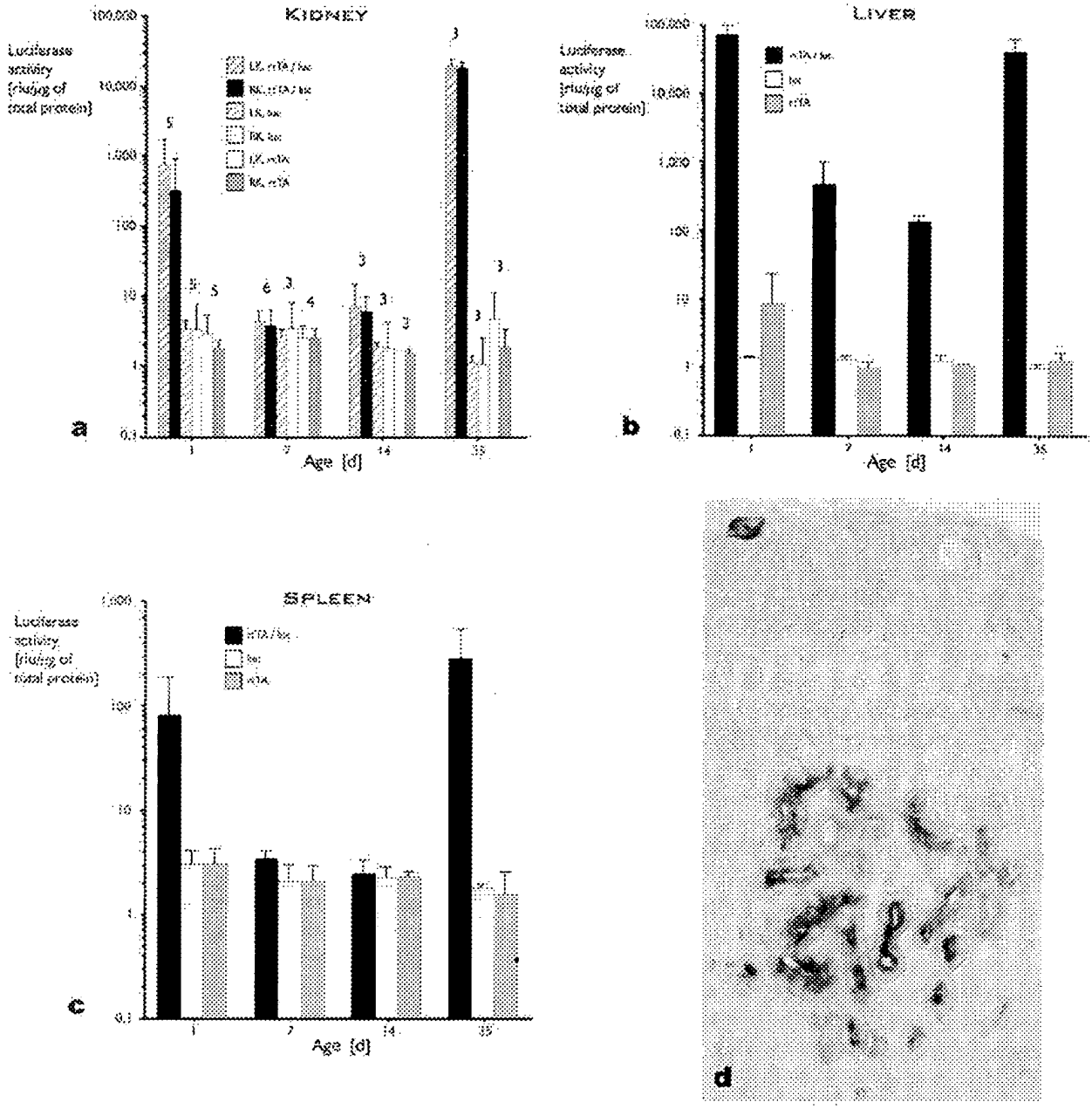

Figure 4. Activity of the reverse transactivator during development. (a through c) Female mice carrying a liver activator protein (LAP) promoter/rtTA2^S-S2 expression cassette (rtTA) were fed doxycycline in the drinking water from 7 d before being mated with male mice carrying a tetracycline-dependent (tet) operator/luciferase expression cassette (luc). Luciferase activity was determined in both kidneys, liver, and spleen of newborn mice and 7, 14, and 35 d after birth. High luciferase activity was observed in the kidneys and liver of newborn double-transgenic (rtTA/luc) mice and 35 d after birth, whereas it was considerably lower in the intermediate time points, suggesting that sufficient levels of doxycycline crossed the placenta, but that not enough doxycycline was provided with the mothers' milk. After weaning, when the offspring began to drink water with doxycycline, the reverse transactivator obviously became activated again. (d) Histological section of a kidney from a newborn mouse with β-galactosidase instead of luciferase as a reporter protein. Double staining for β-galactosidase and alkaline phosphatase, which marks differentiated proximal tubules, demonstrates that the LAP promoter is not active in the nephrogenic zone immediately below the capsule but only in mature proximal tubules. RK and LK, right and left kidney, respectively. Data are presented as mean + SD; the number of mice is given above the bars.

activity has already been seen in the brain, where a reverse transactivator line under the control of the CaMKII-α promoter showed expression in the striatum and septum when placed only shortly on doxycycline; with a longer induction period, expression could also be detected in the cortex and hippocampus (16). We also noticed that even though there was strong

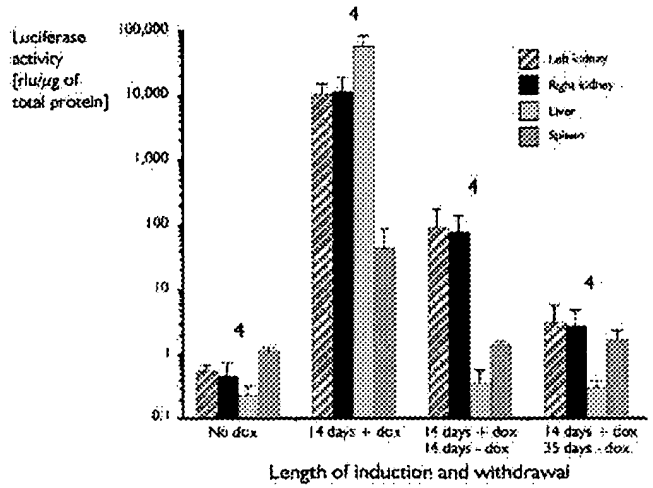

Figure 5. Persistence of reverse transactivator activity. Adult double-transgenic mice containing liver activator protein (LAP) promoter/rtTA2^S-S2 and tetracycline-dependent (tet) operator/luciferase expression cassettes were fed doxycycline in the drinking water for 14 d and then transferred to drinking water without doxycycline. It becomes evident that the activation of the reverse transactivator is reversible, but that it takes longer in the kidney than in the liver before the transactivator returns to its uninduced state. Data are given as mean + SD; the number of mice is given above the bars.

expression in proximal tubules, in many cases, not every nucleus in a given tubular profile was blue. This mosaic pattern of *LacZ* expression has been noted previously (17), but the underlying mechanisms are poorly understood, although it is thought to depend on chromosomal context and transgene copy number (18).

Depending on the kind of study to be conducted, mosaicism represents a more or less serious problem. For example, if one wants to investigate tumor induction, the mosaic inactivation of a tumor suppressor gene upon the induction of Cre recombinase would still permit the development of tumors and might actually be advantageous because it would be possible to perform a genotype-phenotype correlation of normal and malignant tissue in the same organ (the prediction being that in tumors both copies of a tumor suppressor gene are inactivated, whereas in nontransformed tissue one or both alleles are still wild-type). On the other hand, physiologic studies may be severely hindered by mosaicism because wild-type cells would be able to compensate for the cells in which the inactivation event has taken place. Those investigations would therefore only be feasible at a low level of mosaicism.

The rate of induction in the kidney and liver was very fast: we could detect strong reporter gene expression already 1 d after administering doxycycline in the drinking water, which obviously is a convenient method of supplying the chemical. Upon discontinuation of doxycycline, the activity in the liver was reduced to background levels after 14 d of withdrawal. However, transgene expression in the kidney decreased more slowly, reaching background levels after 5-wk withdrawal. The difference between the kidney and liver is most likely the result of the pharmacokinetics of doxycycline in these tissues. Studies with other tetracyclines have demonstrated that higher levels of tetracyclines accumulate in the kidney than in the liver (19,20), which would account for the slower reduction of transgene expression in the kidney compared with the liver. A lower concentration of doxycycline in the drinking water may help to accelerate the return to background levels, although it would entail lower induction levels. The pronounced difference of induction levels between 0.2 and 2 mg/ml of doxycycline in the drinking water (approximately tenfold) was somewhat surprising because *in vitro* the rtTA2^S-S2 reverse transactivator shows full activation already at approximately 1 µg/ml of doxycycline (5). It is likely that the concentration of

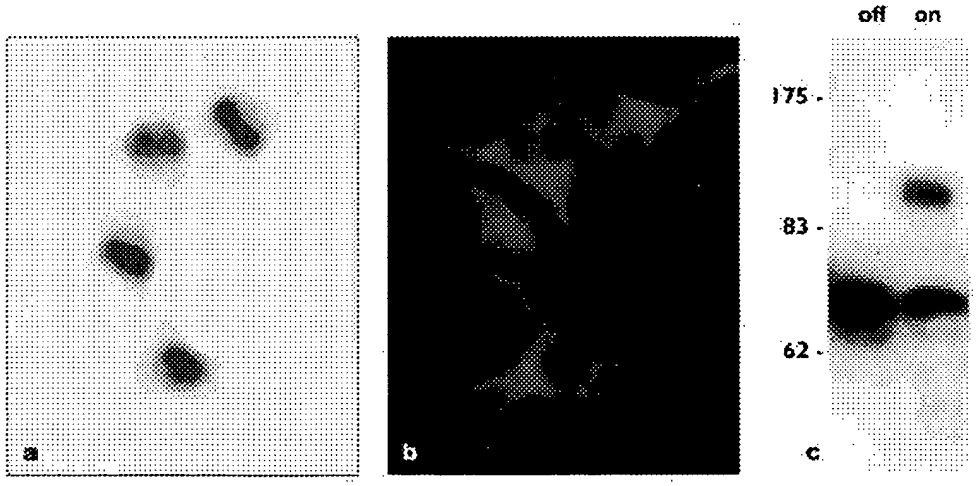

Figure 6. Simultaneous induction of polycystin-2 and β-galactosidase in stably transfected cells. (a through c) A bidirectional expression cassette consisting of the human PKD2 cDNA and a *nLacZ* gene under control of tetracycline-dependent (tet) operator sequences was stably transfected into HeLa cells producing a tTA. Upon induction, the nuclei of transfected cells stained positive for β-galactosidase (a), whereas the HA-tagged polycystin-2 protein was detected in the cytoplasm (b). In the case of polycystin-2, this immunocytochemical finding was subsequently confirmed by Western blot analysis (c; molecular weights are given in kDa).

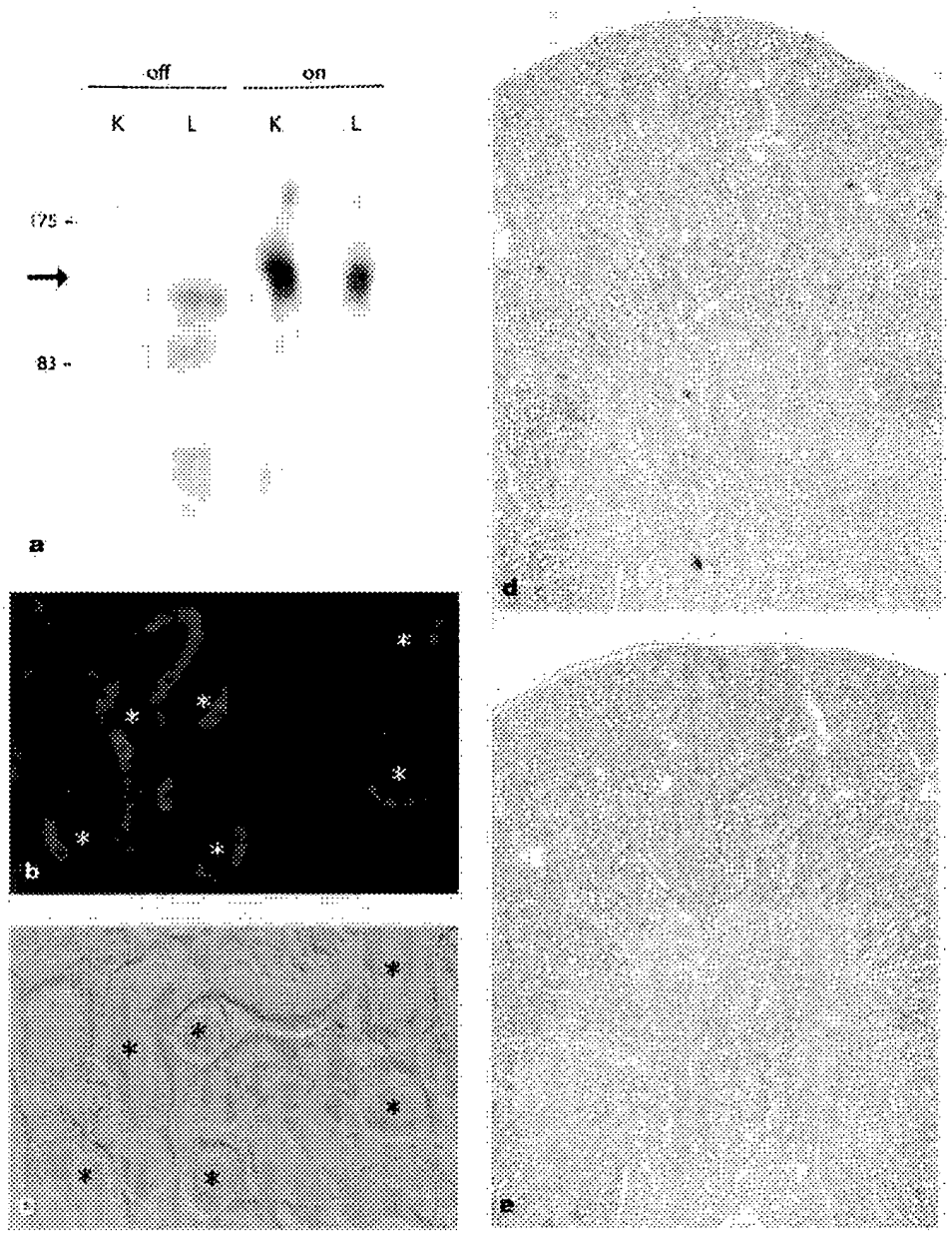

Figure 7. Inducible expression of the human PKD2 cDNA in the rTA^{LAP}-1 mouse line. The bidirectional PKD2/*nLacZ* expression cassette was used to establish transgenic mice, which were then mated with mice containing the reverse transactivator under control of the liver activator protein (LAP) promoter. (a) Western blot analysis was performed with 50 μg of protein prepared from kidney and liver. Only in those mice receiving doxycycline in the drinking water ("on"), polycystin-2 (arrow) can be detected in both the kidney (K) and the liver (L); without doxycycline ("off"), neither organ produces polycystin-2 (the numbers on the left indicate the molecular weight in kDa). (b, c) When kidney sections from double-transgenic mice that had been fed doxycycline in the drinking water were subjected to immunohistochemistry with the anti-HA epitope antibody, polycystin-2 was detected in cortical tubular profiles. Nomarski optics were used to identify these profiles as proximal tubules through the presence of their brush border (asterisks in b and c). (d, e) PAS staining of kidney sections from double-transgenic mice reveals no morphologic changes to nontreated mice (d) even after exposure to doxycycline for 6 mo (e).

doxycycline in the serum and extracellular fluid is much lower than that in the drinking water, possibly in the nanogram per milliliter range, but direct measurements would be necessary to make precise statements. If this explanation is correct, then the intraperitoneal administration of doxycycline may prove advantageous because the issue of uptake via the intestine is eliminated. Clearly, however, data obtained *in vitro* have to be transferred very cautiously to experiments with animals. On the other hand, our withdrawal study illustrates the advantage of the tet-on system in animals. If we had used the original tet-off system, a rapid induction after withdrawal of tetracycline would have been virtually impossible.

Consistent with previous observations that doxycycline can pass readily through the placental barrier (21), we noticed transgene activation in kidneys and livers of newborn mice. It is obvious, however, that sufficient levels of doxycycline do not pass through the mothers' milk, an interpretation based on the only very moderate reporter gene activity at 7 and 14 d postpartum. Therefore, a sustained induction in the postnatal period has to be obtained by intraperitoneal administration of doxycycline.

Finally, we made use of the rTA^{LAP}-1 mouse line to inducibly produce human polycystin-2 in the kidney. Similar to what had been described for a human *PKD1* transgene (22), we had hoped that the expression of the human PKD2 cDNA would lead to the development of cysts. Even after 6 mo, however, no cysts were observed. There may be several explanations for such a result. First, we did not reach high enough levels of polycystin-2 to induce cystogenesis. Second, there is no critical level above which wild-type polycystin-2 would lead to negative effects. Third, expression of the PKD2 cDNA in other nephron segments and not only in the S1 and S2 portions of the proximal tubule would have resulted in cysts. And fourth, as a result of the species difference (we used the human PKD2 cDNA), the cascade toward cyst formation was not turned on. At this point, it is not possible to determine what explanation is the correct one, but it could very well be a combination of these factors.

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JCB Report

Nogo-A expressed in Schwann cells impairs axonal regeneration after peripheral nerve injury

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Injured axons in mammalian peripheral nerves often regenerate successfully over long distances, in contrast to axons in the brain and spinal cord (CNS). Neurite growth-inhibitory proteins, including the recently cloned membrane protein Nogo-A, are enriched in the CNS, in particular in myelin. Nogo-A is not detectable in peripheral nerve myelin. Using regulated transgenic expression of Nogo-A in peripheral nerve Schwann cells, we show that axonal regeneration and functional recovery are impaired after a sciatic nerve crush. Nogo-A thus overrides the growth-permissive and -promoting effects of the lesioned peripheral nerve, demonstrating its in vivo potency as an inhibitor of axonal regeneration.

Introduction

Lesioned central axons successfully regenerate over long distances into peripheral nerves transplanted into the brain or spinal cord (Bray et al., 1987), whereas cultured peripheral axons grown under optimal conditions strictly refuse to invade explants of adult CNS tissue, in particular CNS white matter (Schwab and Thoenen, 1985). This observation suggested for the first time the presence of specific neurite growth-inhibitory factors in the adult CNS (Schwab and Thoenen, 1985; Caroni and Schwab, 1988). Various bioassays pointed to a high molecular weight membrane protein (NI-250, IN-1 antigen) later purified and identified as Nogo-A (Caroni and Schwab, 1988; Chen et al., 2000). Subsequently, several proteins and proteoglycans with neurite growth-inhibitory activity were identified in CNS myelin, including myelin-associated glycoprotein and chondroitin sulfate proteoglycans (Qiu et al., 2000).

To investigate in vivo the inhibitory characteristics of Nogo-A, we generated transgenic mice expressing the rat *nogo A* gene under the inducible control of the Schwann cell–specific *P0* promoter (unpublished data). P0 is the major structural protein of peripheral myelin, and previous studies demonstrated the usefulness and specificity of the *P0* promoter for transgene expression in Schwann cells (Messing et al., 1992).

Results and discussion

Transgenic mice were generated in which a myc-tagged *nogo A* gene was expressed postnatally using the inducible rtTA2 system (Urlinger et al., 2000) under the control of a fusion promoter (*P0Cx*). The *P0Cx* fusion promoter is composed of Schwann cell–specific regulatory elements of the 1.1-kb rat *P0* promoter and of the human connexin 32 gene (*Cx32*; Abel et al., 1999). The 1.1-kb *P0* promoter alone was often used, but transgene expression was inconsistent (Feltri et al., 1999). For high expression in mice, a Schwann cell–specific intron from the *Cx32* gene was added after the *P0* promoter. By inducing the expression of *nogo A* only after birth, we could avoid possible effects of Nogo-A protein on the development of the PNS. Transgenic lines were made that express the rtTA2 or the responder, a bidirectional tetO promoter (pBI-3 construct; Baron et al., 1995) directing *nogo A* and *lacZ* transcription (Fig. 1 A). rtTA2 is a second generation rtTA system, which requires low doses of doxycycline for activation and has almost no background activity (Urlinger et al., 2000). To test the pBI-3-*nogo A*, we transfected it into HeLa cells stably transfected with a tTA construct, which activates transcription from tetO promoters in the absence of doxycycline (Gossen and Bujard, 1992). The cells expressed

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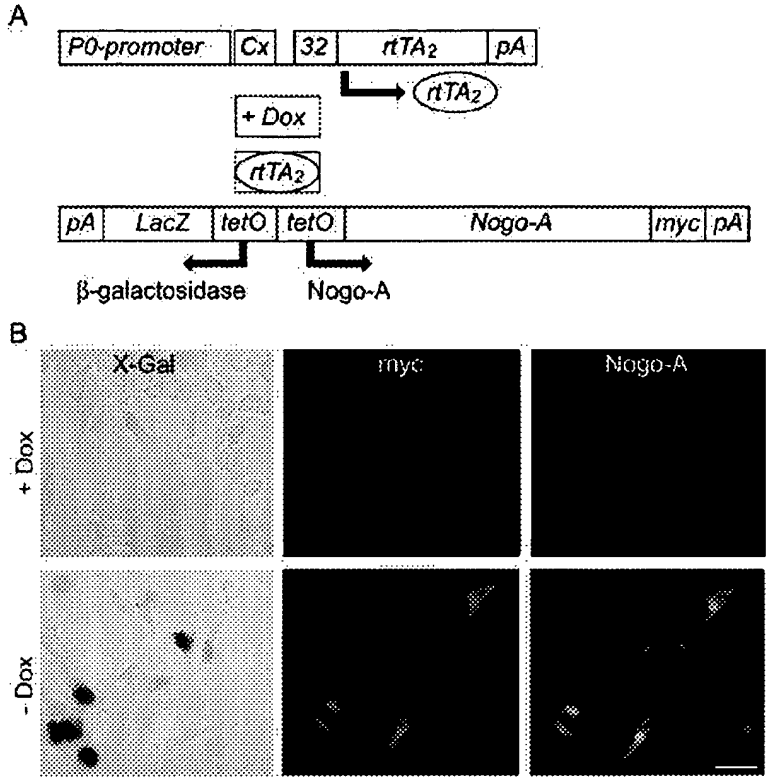


Figure 1. **Regulated expression system for the *nogo A* transgene, and in vitro expression of β-galactosidase and *nogo A–myc*.** (A) Strategy used to obtain Schwann cell–specific doxycycline-regulated transgene expression. Transgenic mice carrying the *rtTA2* gene driven by the rat 1.1-kb *P0* promoter fused to 320 bp of the connexin 32 fragment were crossed with mice carrying the bidirectional *tetO* promoter inducing *nogo A–myc* and *lacZ* transcription when doxycycline is added. (B) TetO-*nogo A/LacZ* transfected into tTA HeLa cells (expression is activated by removal of doxycycline in the tTA system) showed no X-Gal, myc, or Nogo-A staining when doxycycline was in the medium (+Dox). When doxycycline was withdrawn (−Dox), the cells showed expression of X-Gal, myc, and Nogo-A. Bar, 25 μm.

both β-galactosidase and *nogo A* when doxycycline was omitted from the medium (Fig. 1 B).

Several founder mouse lines were obtained from pBI-3-*nogo A* and *P0Cx*-rtTA2 constructs and the strongest expressing lines were used. Single transgenic mice were mated to produce the Nogo-A–expressing double transgenic mice. The system was not leaky because X-Gal staining was only found in sciatic nerves of doxycycline-treated double transgenic mice. Nogo-A expression was induced at birth by feeding the mothers with doxycycline. The efficiency and specificity of the promoter was assessed in three ways. (1) X-Gal staining of the sciatic nerve showed large numbers of labeled cells (Fig. 2 A). No labeling was present in the spinal cord (unpublished data). Immunohistochemistry for (2) Nogo-A and (3) myc showed Nogo-A– and myc-positive cells and myelin structures in sciatic nerves exclusively of the double transgenic animals under doxycycline. Nogo-A, colocalized with the specific marker of Schwann cells S-100, was found in the Schwann cell bodies and in the outer and inner loops of the myelin, similar to that described for Nogo-A in oligodendrocytes and in myelin of the CNS (Fig. 2 C) (Huber et al., 2002). Two Nogo-A transgenic mouse lines, Tg11 and Tg16, were selected and compared using the X-Gal staining; the mice of the Tg11 line showed stronger staining than those from Tg16 (Fig. 2 A). At least 20% of the Schwann cells in line Tg16 and >45% of the Schwann cells in line Tg11 expressed a high level of the transgene (Fig. 2, B and C). The overall level of expression of the transgene in the sciatic nerve (Tg11) was about one third that of endogenous Nogo-A in the optic nerve (CNS) of the same animals (unpublished data).

Modifying the components of peripheral myelin could disturb the myelin structure (Siconolfi and Seeds, 2001). At the light and electron microscopic level, the Schwann cells, axons, and myelin of Nogo-A–expressing peripheral nerves appeared normal (Fig. 3, A–C). The transgenic mice developed normally and their gait was normal, as indicated by footprint analysis (overlapping footprint patterns for control and transgenic mice). Motor coordination and balance were tested on the rotarod (Fig. 3 D) at 10 wk of age and no deficits were recorded in transgenic mice. The animals were also indistinguishable from control littermates (single transgenics for *P0-rtTA* or *nogo A/lacZ* only or wild types), using the narrow beam test (unpublished data).

To test the influence of Nogo-A expression on axonal regeneration in the PNS, freeze-crush lesions of the sciatic nerve on 8–12-wk-old mice were performed. All motor (and presumably sensory) axons were lesioned, as indicated by the failure of Fluorogold tracer to retrogradely label motoneurons when applied 0–2 d after the crush (n = 12; unpublished data). Neurological recovery was analyzed by the sciatic functional index (SFI)* (de Medinaceli et al., 1982), which characterizes hindlimb use and foot and toe positions, and by the toe pinch reflex (Siconolfi and Seeds, 2001), a simple sensory motor reflex (Fig. 4). The number of successfully regenerating motoneuron axons was then determined by a retrograde tracing and a direct immunostaining of the axons distal to the lesion (Sagot et al., 1998). For all behavioral tasks and operations, double transgenic mice (Tg) and control littermates

*Abbreviations used in this paper: PFA, paraformaldehyde; SFI, sciatic functional index.

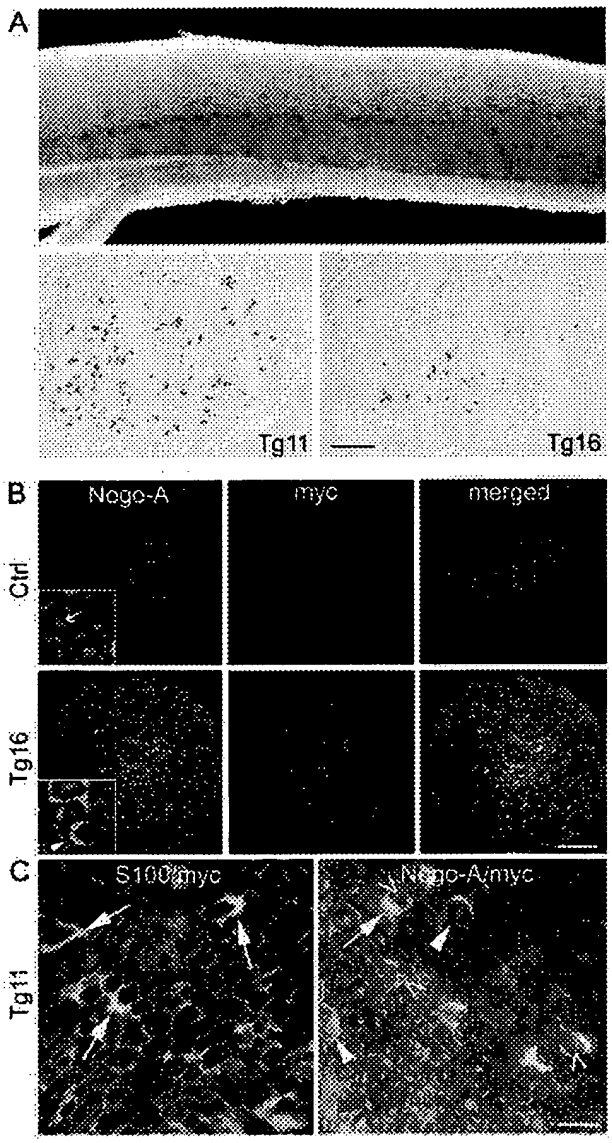


Figure 2. **In vivo expression of the transgene in the sciatic nerve.** (A) X-Gal staining of a sciatic nerve whole mount of a 3-mo-old double transgenic mouse of Tg16 results in blue-labeled Schwann cell nuclei (nuclear localization signal preceding *lacZ*). Cross sections of 7-wk-old mouse sciatic nerves show more X-Gal staining in the Schwann cells of Tg11 than of Tg16. (B) Colocalization of myc with Nogo-A protein. At low magnification, cross sections of P25 sciatic nerves of control mice (Ctrl) show weak, mostly axonal (insert, thin arrow) staining of Nogo-A and no myc staining. In the transgenic animal (Tg16) the Nogo-A level is higher and Nogo-A is colocalized with myc (merged). The insert shows Nogo-A in axons and additionally in the outer myelin loops (arrowheads). The immunological signals for Nogo-A were stronger than those for the myc tag in this study. (C) Colocalization of myc with the Schwann cell marker S-100 and of myc with Nogo-A. In double immunolabeled sections of Tg11, S-100/myc (left) and Nogo-A/myc (right) are colocalized in the Schwann cell bodies (arrows), as well as in the outer (closed arrowheads) and inner (open arrowhead) loops of myelin, similar to the localization of Nogo-A in oligodendrocytes and CNS myelin (Huber et al., 2002). Bars: (A and B) 60 μm; (C) 20 μm.

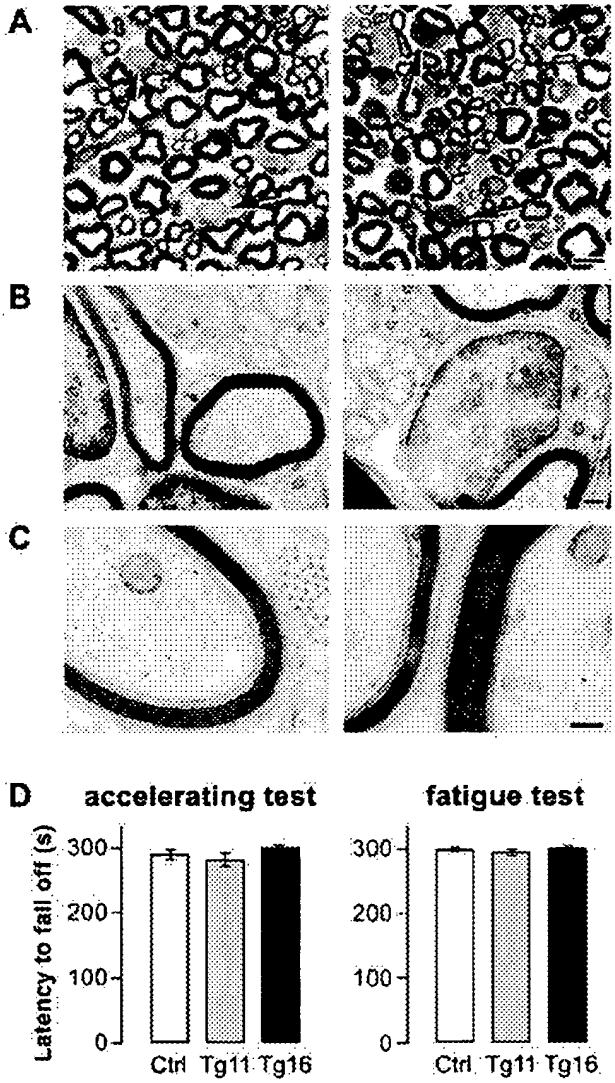
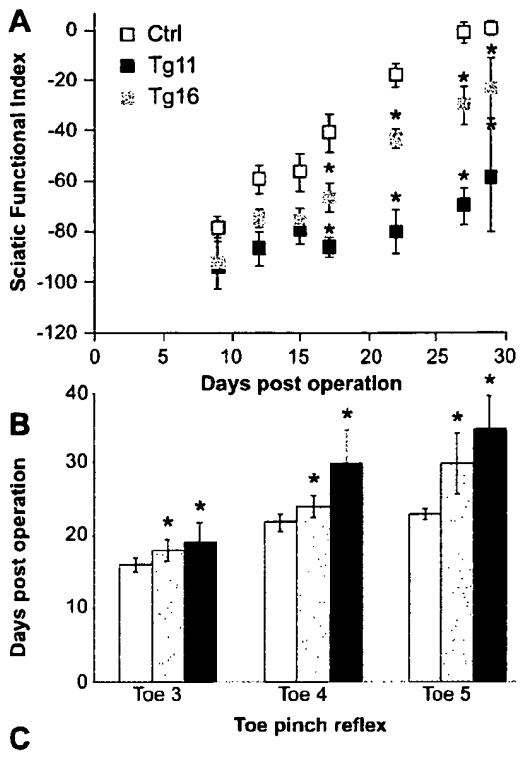


Figure 3. **Nogo-A transgenic animals have a normal sciatic nerve histology and normal motor behavior before lesion.** (A) Semi-thin sections of sciatic nerve from a 7-wk-old control (Ctrl) mouse and an age-matched Nogo-A transgenic (Tg11) stained with toluidine blue show normally myelinated large and small caliber axons. Schwann cells (arrows) are present and normal. (B) Electron micrographs show no detectable cytoplasmic abnormalities in myelinating and nonmyelinating Schwann cells in Ctrl and Tg11. (C) No differences were seen for the myelin spacing of Ctrl and Tg11 Schwann cells. (D) Behavioral assessment. 10-wk-old mice (Ctrl, Tg11, and Tg16) were tested on a rotating rod (rotarod) on two consecutive days. No differences were found in the latency to fall off from the rotarod, neither in the acceleration test (left) nor in the fatigue test (right). The means (±SEM) of control (Ctrl; $n = 23$), line 11 (Tg11; $n = 7$), and line 16 mice (Tg16; $n = 13$) are presented. Bars: (A) 12 μm; (B) 1.2 μm; (C) 0.25 μm.

(Ctr), having no transgene or a single transgene (*P0Cx-rtTA2* or *nogo A/lacZ* only), were used. All animals received doxycycline from birth until the end of the experiments.

The SFI evaluates crucial aspects of locomotion involving recovery of hindlimb sensory and motor function. Nonlesioned animals have an SFI of zero (±10). Both transgenic



C

	Toe 3	Toe 4	Toe 5
control	100 %	91 %	75 %
Tg 16	100 %	80 %	60 %
Tg 11	75 %	25 %	25 %

Toe pinch reflex recovery 30 days post operation

Figure 4. **Two independent Nogo-A transgenic mouse lines show a decrease in behavioral recovery after a sciatic nerve crush as compared with controls.** (A) Recovery of locomotor function after sciatic nerve crush determined by the SFI. Nonlesioned animals have an SFI of zero. 28 d after the lesion, functional recovery was poor and the deficit long lasting in Tg11, delayed and incomplete in Tg16, and complete in the control mice. Means (±SEM) of control (Ctrl; $n = 10$), line 16 (Tg16; $n = 5$), and line 11 (Tg11; $n = 3$) animals are shown. *, two-tailed Mann Whitney test, $P < 0.05$ compared with controls. (B) Recovery of the toe pinch reflex is delayed in Tg11 and Tg16. Histogram shows the time in days after sciatic nerve crush at which initial toe pinch reflex was elicited for toe 3, toe 4, and toe 5. Mice from both lines show a delay in recovery, which is more pronounced in Tg11 than Tg16. The means (±SEM) of control (Ctrl; $n = 12$), line 11 (Tg11; $n = 4$), and line 16 (Tg16; $n = 5$) animals are presented. *, two-tailed Mann Whitney test, $P < 0.05$ compared with control. (C) Table of the distribution of animals that showed toe pinch reflex response 30 d after the operation.

and control mice showed normal SFI values before the lesion. Sciatic nerve crush produced a massive disability manifested by a negative SFI that returned to zero as the sciatic axons regenerated. Control mice recovered with a typical time course of 2–3 wk (Fig. 4 A), in line with published data (Chen and Bisby, 1993). For the two Nogo-A transgenic lines, recovery was significantly delayed. Tg11 mice showed an overall poor recovery with large deficits persisting 30 d after the lesion. SFI recovery of Tg16 mice was better than of Tg11, but still significantly slower and less complete than in the control mice (Fig. 4 A).

In the toe pinch reflex, before sciatic nerve crush, all mice showed equal sensitivity; a pinch of toe 3, 4, or 5 (tested separately) reliably induced a rapid retraction of the leg (digits 1 and 2 are innervated by the saphenous nerve and were therefore not tested). After the crush, the response was totally abolished in all animals. The time taken for the injured hindlimb to show any degree of a response to the stimulus was noted. Digit function recovery occurred in a medial to lateral direction in all mice (Fig. 4 B). The recovery was monitored over 30 d, and in many of the transgenic mice the sensitivity of the last two digits did not appear. The toe pinch reflex values from those animals were extrapolated from the recovery curve. The percentage of animals responding 30 d after the lesion was calculated (Fig. 4 C). For the digit 4 of control animals, 91% showed sensitivity (75% for digit 5) compared with 80% for Tg16 (60% for digit 5) and 25% for Tg11 (25% for digit 5) (Fig. 4 C). Thus, the Nogo-A transgenic mice showed a significant delay in the recovery of the toe pinch reflex.

Spinal motoneurons were retrogradely labeled with Fluorogold 7, 14, and 28 d after the crush from a site 7 mm distal to the lesion (Fig. 5, A and C). Fluorogold was chosen as a marker because it is a long-lasting and nondiffusible tracer that undergoes rapid retrograde axonal transport. Nonlesioned transgenic mice had the same number of motoneurons as wild-type mice. A time course with wild-type mice was established to determine the time at which the transgenic animals were to be traced after the lesion. By 5 d, no motoneuron axons had reached the injection site. Between 7 and 14 d, a gradual increase in the number of retrogradely labeled motoneurons was seen and the peak number of motoneurons was reached 17 d after the lesion. Therefore, 7 and 14 d after the lesion were chosen to analyze the motor axonal regeneration, and a later time point (28 d) to analyze axon numbers at a time when functional recovery in control animals was complete. 7 and 14 d after a sciatic nerve lesion, a significantly lower number of motor axons had grown 7 mm past the lesion in transgenic animals compared with control animals (Fig. 5 B), indicating a slower regeneration of the motoneuron axons. 28 d after the lesion, only a small difference was noted in both transgenic groups compared with the control group, indicating that almost all the axons had reached the injection site. The discrepancy between this last result and the behavioral data (Fig. 4) could be due to the fact that 28 d after the lesion, most of the motoneuron axons of the transgenic mice had reached the injection site 7 mm distal of the lesion but not the muscle target (~15 mm away from the lesion), or not the appropriate target. Correct locomotion is also dependent on regeneration and correct targeting of sensory axons, which were not studied yet.

Finally, regenerating axons in the sciatic nerve were directly assessed using GAP-43 and neurofilament (SMI-32) markers. GAP-43 stains mainly growing axons. SMI-32 recognizes nonphosphorylated neurofilaments, described as being more abundant than the phosphorylated form in regenerating axons of the lesioned sciatic nerve (Pestronk et al., 1990; Tsuda et al., 2000). 7 d after the lesion, the labeled axons were counted on cross sections 4 mm distal to the lesion on randomly photographed sections. In contrast to intact nerves, most of these regenerating fibers were thin, fine cali-

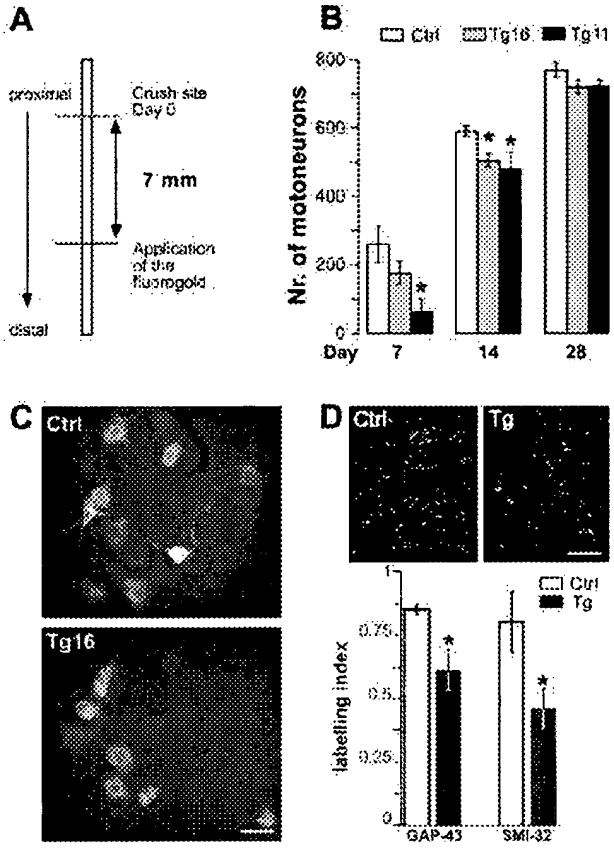


Figure 5. **Retrograde tracing and regenerating axons.** (A) 7, 14, and 28 d after a sciatic nerve crush, the nerve was cut 7 mm distal to the lesion and crystals of the retrograde tracer Fluorogold were applied to the cut nerve. (B) 7 and 14 d after the lesion, the number of retrogradely labeled motoneurons is significantly smaller in the two transgenic lines (Tg11 and Tg16) compared with control animals, indicating a delay in axonal growth. 28 d after the lesion, no significant differences were seen; almost all the axons of the three mice groups had reached the injection site. *, unpaired two-tailed *t* test, $P < 0.05$ compared with control. The means (±SEM) are represented, $n = 5$–8 in each group. (C) Sections through the ventral horn of the spinal cord show a smaller number of labeled motoneurons in Tg16 versus control (Ctrl) animals 14 d after the lesion. (D) Growing axons were directly labeled 4 mm distal to the lesion using antibodies against GAP-43 and the nonphosphorylated neurofilament marker SMI-32. Cross sections (top) were photographed at 630× and all the GAP-43–labeled axons were counted. Histograms (bottom) show the ratio of the axonal counts per section normalized to the highest count, setting the best animal at one. The animals from the transgenic group (Tg; $n = 6$) show significantly less axons stained with GAP-43 or SMI-32 than those from the control group (Ctr; $n = 5$). The means (±SEM) are represented. *, unpaired two-tailed *t* test, $P < 0.05$ compared with control. Bars: (C) 25 μm; (D) 13 μm.

ber fibers. Almost no debris was seen with those two markers. Compared with axon numbers of nonlesioned sciatic nerves, regenerating nerves of control animals had ~40% of the axons (unpublished data). A significantly lower axon number was found in the regenerating sciatic nerves of the transgenic animals using both markers (Fig. 5 D).

Taken together, these data show that postnatal expression of Nogo-A in Schwann cells results in a significant delay in axon regeneration in the denervated adult mouse sciatic nerve. The selective postnatal expression prevented possible effects of Nogo-A on the development of Schwann cells and axons, or on the process of myelin formation. The doxycycline treatment cannot be responsible for the observed differences in regeneration because all the animals, controls and transgenics, were treated with the drug. It is also unlikely that the reporter *lacZ* had a negative effect because myelin and Schwann cell morphology was normal, and, previously, the reporter had been used in Schwann cells without any signs of toxicity (Arroyo et al., 1998).

Efficient and successful regeneration in peripheral nerve is influenced by Schwann cell–released neurotrophic factors, extracellular matrix molecules, and basement membrane components. The expression of Nogo-A slows down the rate of axon growth compared with that seen in a normal denervated sciatic nerve. The difference in the regenerative potential observed between the two *nogo A*–expressing mouse lines (Fig. 4) is probably related to their difference in expression levels of *nogo A* (Fig. 2 B). *P0* promoter activity is also known to be temporarily decreased distal to a lesion in the context of Schwann cell dedifferentiation (Gupta et al., 1988), thus possibly creating a window of increased opportunity for regenerating axons, although significant levels of Nogo-A protein may still be present on myelin debris. *P0* promoter is known to be strongly induced starting 7 d after peripheral nerve lesion when the remyelination takes place (Gupta et al., 1988), and thus could represent an impediment for late growing axons. All these data strongly indicate that Nogo-A is a potent neurite growth inhibitor that can override multiple strong regeneration-enhancing factors known to be present in lesioned peripheral nerves.

Nogo-A, constitutively present in CNS myelin, biases the balance of growth-promoting and -inhibitory factors toward inhibition of regeneration, leading to a restriction of plasticity and functional recovery. The inhibitory property of Nogo-A is further demonstrated by enhanced regeneration, compensatory sprouting, and functional recovery of lesioned CNS tracts resulting from in vivo application of the monoclonal antibody IN-1 (Schnell and Schwab, 1990; Thallmair et al., 1998; Merkler et al., 2001; Papadopoulos et al., 2002). Similar results were obtained with autoantibodies against myelin (Huang et al., 1999) and antibodies against specific regions of the Nogo-A molecule (unpublished data). All these results suggest that blockade of Nogo-A signaling by antibodies, receptor-blocking reagents (GrandPre et al., 2002), or drugs acting at the postreceptor level represent exciting experimental approaches for therapies of CNS injuries, including spinal cord or brain trauma and stroke.

Materials and methods

Plasmids for construction of the transgenic mice

For the generation of rtTA2-expressing mice, a 1.1-kb *P0* rat promoter followed by a 350-bp portion of the human connexin 32 gene (30 bp of the promoter, exon 1B, intron 1, and 20 bp of exon 2) was placed upstream of the full-length *rtTA2* gene. A β-globin transcription termination signal was added to the 3′end. To generate the *tet-O–nogo A–lacZ* construct, a bidirectional tetO promoter, consisting of a human cytomegalovirus minimal promoter linked to the tet operator sequences was used. A 3.5-kb DNA fragment encoding *nogo A* with myc and Xpress tags at the COOH terminus was placed downstream of the tetO promoter in pBI-3 (Baron et al., 1995) (Fig. 1

A). Restriction fragments containing each transgene were isolated from vector sequences and prepared for microinjection into fertilized oocytes

Controlling the doxycycline-regulatable expression of *nogo A* in HeLa cells

HeLa cells, stably expressing the tTA construct, were transfected with pBI-3-*nogoA* construct using FuGENE 6 transfection reagent (Roche). 3 μg/ml doxycycline solution was added to the culture medium for 24 h. The cells were fixed for 15 min in 4% paraformaldehyde (PFA) and immunostained for myc and Nogo-A, and the activity of β-galactosidase was assessed. For β-galactosidase staining, the cells were incubated for 1 h at 37°C in a solution containing 1 mg/ml X-Gal (Roche), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.1% Triton X-100, and 2 mM $MgCl_2$ in PBS. For immunofluorescence, the cells were permeabilized with 0.1% Triton X-100 in PBS and blocked with 10% FCS. Mouse anti-myc antibodies (clone 9E10; Sigma-Aldrich) were incubated simultaneously with the rabbit anti–Nogo-A antiserum 472 (Chen et al., 2000) for 30 min at RT. Rabbit antibodies were visualized by anti–rabbit FITC-conjugated secondary antibody, and mouse antibodies by anti–mouse TRITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories).

Mouse breeding and genotyping

All transgenic lines were created using the hybrid strain B6D2F1. For both constructs, founder lines were crossed for two to six generations into the inbred strain C57BL/6. Double transgenic mice were obtained from crosses between mice heterozygous for each transgene. The line with the strongest expression of the transactivator (*Tg-[P0Cx-rtTA2] 693 Zhz*) and of two independent reporter lines (*Tg-[Nogo/LacZ] 728* and *732 Zhz*) were crossed, yielding the double transgenic lines Tg11 and Tg16. The genotypes of the mice were determined by PCR analysis of genomic DNA isolated from mouse tails. Primers 5′-CACGGCGGACAGAGCGTACAG-3′ and 5′-CCGAATTCACCATGTCTAGACTGG-3′ were used to amplify a 600-bp fragment from the rtTA2 construct, and primers 5′-CCTGCTGCATCTGAGCCTGTG-3′ (first exon of *nogo A*) and 5′-ACAGGTGCTACTACTGACATCTG-3′ (second exon of *nogo A*) to amplify a 548-bp fragment from the cDNA for the *tetO–nogo A* construct. Transgene expression was induced at birth by replacing normal drinking water with 5% sucrose containing doxycycline (2 mg/ml). The mice were kept under doxycycline until the end of the experiments.

Whole mount sciatic nerve preparation and immunofluorescence microscopy

Sciatic nerves of control and *nogo A* transgenic animals were dissected and fixed by immersion in 4% PFA for 20 min on ice. For β-galactosidase staining, the whole sciatic nerve was incubated overnight at 37°C in X-Gal solution. After staining and photographing, the tissue was washed in PBS, postfixed for 1 h in 4% PFA, and then frozen in the same block. Cryostat sections (20 μm) were cut. For immunofluorescence, the sections from wild-type and transgenic animals were processed on the same slide by permeabilization with 0.03–0.3% Triton X-100 in PBS and blocked by 1% BSA or 2% rat serum. Cy3-conjugated mouse anti-myc antibodies (clone 9E10; Sigma-Aldrich) were incubated simultaneously with a rabbit anti–Nogo-A antiserum 472 or a rabbit anti-S100 antibody (Dako). The sections were analyzed using a confocal ZEISS LSM 410 microscope or a ZEISS Axiophot microscope equipped for epifluorescence.

Ultrastructural analysis

Mice were deeply anesthetized with pentobarbital and transcardially perfused by Ringer solution followed by 2% glutaraldehyde and 2% PFA in 0.1 M phosphate buffer. All fixative and buffer solutions were supplemented with 2 mM $CaCl_2$. Sciatic nerves were removed, placed in fresh fixative for 3 h, and then placed in cacodylate buffer overnight at 4°C. Tissues were postfixed in 2% OsO_4 for 2 h, serially dehydrated, and embedded in Epon. Semi-thin sections were stained with toluidine blue and viewed under an Olympus microscope. Ultrathin sections (90 nm) were analyzed using a ZEISS EM 902.

Surgery

Mice (16–26 g) were deeply anesthetized by intraperitoneal injection of fentanyl citrate (0.0189 mg/100 mg), fluanisone (0.6 mg/100 mg; Hypnorm; Jansen Biochemica), and midazolam (0.6 mg/100 mg; Dormicum; Hoffmann-La Roche). The sciatic nerve was exposed in the upper thigh and freeze crushed with watch-maker forceps that had been previously cooled in liquid nitrogen for 30 s. The epineurium remained intact. The crush site was marked with charcoal powder. The experiments were performed in conformation with the Swiss animal protection laws and were approved by the Cantonal Veterinary Department of Zurich.

Behavioral tests

Rotarod. Animals were tested for two consecutive days on a rotating rod; the first day for the acceleration test and the second day for the fatigue test. The acceleration test was performed by placing a mouse on the revolving rod. Once balanced, the rod was accelerated from 4 to 40 rpm over a 300-s period. The fatigue test was performed at a fixed speed of 40 rpm for 300 s. For both tests, the latency for the mouse to fall off the rod was determined with a cut-off of 300 s. The mice were given five trials with a 20-min rest interval between each trial. The average time on the rod for each mouse was used for analysis.

SFI. After first pressing their hind paws onto an ink pad, the animals were tested along a confined 60-cm-long walkway lined with plain white paper. The tracks were analyzed according to the empirical equation determined by de Medinaceli et al. (1982).

Toe pinch reflex. Recovery of pain sensitivity was tested on awake mice by lightly pinching the most distal portion of the last three digits of the lesioned hind limb with forceps. The first day after the lesion at which foot withdrawal was restored was recorded.

Motoneuron tracing and histological analysis

Motoneurons tracing. 7, 14, and 28 d after surgery, crystals of Fluorogold (Molecular Probes) were applied 7 mm distal to the crush lesion onto the cut nerve. 48 h later, mice were deeply anesthetized with pentobarbital and perfused with 4% PFA. Spinal cords were removed and processed for cryosectioning as described by Sagot et al. (1998). Cryostat serial sections (30 μm) were viewed under fluorescence illumination, and Fluorogold-labeled motoneurons, identified by size, shape, and location in the ventral horn, were counted on every section.

Regenerating axons. 7 d after surgery, sciatic nerves were removed, fixed as described above, and cut transversally 4 mm distal to the lesion. After permeabilization with ethanol/acetic acid (95:5; 15 min), the sections (15 μm) were stained with a rabbit antiserum against GAP-43 (Chemicon) or with a mice neurofilament antibody, SMI-32 (Sternberger Monoclonals Inc.). Areas of 10 μm^2 were randomly photographed from the two or three fascicles of each nerve at 630×, and all labeled axons were counted by two different, blinded observers.

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